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(54) Title: LIGASE CHAIN REACTION METHOD FOR DETECTING SMALL MUTATIONS

(57) Abstract

The present invention involves methods of detecting small mutations in target nucleic acid using the Ligase Chain Reaction (LCR™). Detectable small mutations include single base deletions, insertions and changes, as well as multiple mutations (deletions, insertions and changes) where the size of the mutation is less than about 15 % of the average probe length. The methods include high concentrations of monovalent cations, especially Na^+, K^+ and NH_4^+; and/or relatively high initial mixing temperatures. The methods also include probe designs having deliberate mismatches with respect to target, the mismatches being near the 5' ends. Also claimed are several sets of probe sequences for detection of mutations associated with cystic fibrosis.

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LIGASE CHAIN REACTION METHOD FOR DETECTING SMALL MUTATIONS

This application is related to U.S. application Serial No.07/860,702, filed March 31, 1992, pending; and to U.S. application Serial No.07/634,771, filed January 9, 1991, pending, which is a continuation-in-part of application U.S. Serial No. 07/470,674, filed January 31, 1990, now abandoned. The latter two mentioned applications form the basis of EP-A-0 439 182, published on July 31, 1991. The entire disclosure of each of the above-mentioned applications is incorporated herein by reference.

BACKGROUND

This invention relates to methods of amplifying target nucleic acids and, particularly, to methods of detecting small mutations using the Ligase Chain Reaction (LCR™) to selectively amplify the genomic region of interest.

In the target amplification technique known as LCR™, two primary (first and second probes) and two secondary (third and fourth) probes are employed in excess. The first probe hybridizes to a first segment of the target strand and the second probe hybridizes to a second segment of the target strand, the first and second segments being contiguous so that the primary probes abut one another in 5' phosphate-3' hydroxyl relationship and so that a ligase can covalently fuse or ligate the two probes into a fused product. In addition, a third (secondary) probe can hybridize to the first probe and a fourth (secondary) probe can hybridize to the second probe in a similar abutting fashion. Of course, if the target is initially double stranded, the secondary probes will also hybridize to the target complement in the first instance. Once the fused strand of primary probes is separated from the target strand, it will hybridize with the third and fourth probes which can be ligated to form a complementary, secondary fused product. In order to understand LCR™ and the improvements described herein, it is important to realize that the fused products are functionally equivalent to either the target or its complement. By repeated cycles of hybridization and ligation, amplification of the target sequence is achieved. This technique is described more completely in published EP-A-320 308; and in published EP-A-439 182.

Genetic mutations can result from deletions, insertions or changes in one or more bases in the genetic code. In many cases, the cause of a particular illness or disease results from a single or a limited number of mutations or polymorphisms in the genome of the affected individual. When the number of polymorphisms is manageable, determination of the genetic constituency of the individual can be useful in diagnosing

the illness or disease. Some diseases or conditions (*e.g.* sickle cell anemia, phenylketonuria, Tay-Sachs disease, medium chain acyl CoA dehydrogenase deficiency, cystic fibrosis) can manifest themselves as a point mutation of a single base in the DNA. Others (*e.g.* Duchennes Muscular Dystrophy (DMD), and thalassemias) can manifest themselves by short alterations or deletions in one or a relatively small number of exon locations. It will be recognized that the few examples given above are by no means exhaustive, and that there are hundreds or thousands of other disease related mutations or conditions which can be detected using the methods of the invention.

Mutations have been detected in the prior art by tedious and time consuming processes requiring amplification of the relevant genomic region, followed by immobilization of the amplified DNA on a solid membrane and probing the membrane with allele specific probes. This method was improved upon in a procedure known as "reverse dot blot". This procedure is described in Sakai, et al., *Proc. Natl. Acad. Sci. USA*, 86:6230-6234 (1989) and involves the prior immobilization of allele specific probes to locations on a membrane. The amplified sample is then contacted with the membrane to allow hybridization, which will occur only at specific locations or "dots" so the result can be directly read from the membrane.

Other researchers have demonstrated the ability to detect certain mutations by a method referred to as ARMS, which stands for Amplification Refractory Mutation System. For example, see EP-A-0 322 435, assigned to Imperial Chemical Industries, PLC, ("ICI"); Newton,et al, *Nucl. Acids Res.* 17(7):2503-2516 (1989); Newton, et al, *J. Med. Genet.*, 28:248-251 (1991): and Lo, et al, *Nucl. Acids Res.* 19(13):3561-3567 (1991). The methodology of ARMS may be characterized as allele specific PCR, optionally modified by using an additional mismatched base pair. Primers are prepared to amplify the region of a point mutation so that at least one primer of the pair terminates its 3' end at the site of the mutation. Thus, the primer is designed to be perfectly complementary to the normal (or mutant) allele, and to be mismatched at the 3' terminus with respect to the mutant (or normal) allele. Depending on the nature of this mismatch, it alone may be sufficient to cause the primer to be "refractory" to amplification on the mutant (or normal) gene. However, in other cases, particularly when the mismatch results in a pyrimidine/purine mismatch (*i.e.* G/T, T/G, A/C or C/A), the specificity can be improved by introducing an additional mismatch a few bases from the 3' end, thus making the primer even more refractory to amplification in the mutant (or normal) gene.

K. B. Mullis, a co-inventor of the polymerase chain reaction (PCR), has described an improved method in *PCR Methods and Applications*, 1(1):1-4 (1991). The improved process comprises preventing the extension reaction from beginning until the temperature is high enough to discourage binding of non-perfectly hybridized

primers. This can be accomplished, for example, by adding the polymerase to the other components while the temperature remains high from an initial denaturing step. Mullis indicates that permitting the reaction vessels to cool before adding the polymerase can cause non-specific primer binding and extension, resulting in false positive results. This procedure is referred to as "hot start" PCR.

Barany, *Proc. Natl. Acad. Sci. USA* **88**:189-193 (1991) provides a discussion regarding the detection of sickle cell using LCR. In addition, the aforementioned EP-A-439 182 includes some discussion of using "gap filling" LCR™ to detect point mutations which occur in the gap or at the location of the "stop base". While these disclosures addresses a similar problem as that faced by applicants, the solutions differ considerably.

Wu, et al, in *Genomics*, 4:214-228 (1991), disclose the use of relatively high NaCl concentration (200 mM) to suppress undesired target independent blunt-end ligation in a ligase chain reaction. However, the ligase used by these researchers was T4 DNA ligase, which is not thermostable and not of bacterial origin.

Mutations known to be associated with cystic fibrosis have been published in numerous sources, including EP-A-0 497 527, Zielenski, et al, *Genomics*, **10**:214-228 (1991), Kerem, et al, *Science*, **245**:1073-1080, (1989), Riordan, et al, *Science*, **245**:1066-1073, (1989), Rommens, et al, *Science*, **245**:1059-1065, (1989), Cutting, et al, *Nature*, **346**:366-368 (1990), Dean, et al, *Cell*, **61**:863-870, (1990), Kobayashi, et al, *Am. J. Hum. Genet.*, **47**:611-615 (1990), Kerem, et al, *Proc. Natl. Acad. Sci. USA*, **87**:8447 (1990), Vidaud, et al, *Human Genetics*, **85**(4):446-449 (1990), and White, et al, *Nature*, **344**:665-667 (1990).

In spite of the foregoing references, there remains a need to quickly and reliably detect small mutations using the ligase chain reaction. Moreover, while detection of certain mutations has been achievable by known methods, the reaction conditions have generally required specific optimization for each different probe set and target. When multiplex LCR™ is used, however, the conditions cannot be changed for each probe set. Thus, there exists a need in the art for methods which increase the "flexibility" or "dynamic range" of each probe set. The present invention addresses this need, particularly as concerns the detection of cystic fibrosis, although it is applicable to other disease related mutations or conditions as well.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides a method of detecting a small mutation in a target nucleic acid the sequence of which is known, said method including: (a) providing an excess of at least two sets of two probes, the 3' end of a first upstream probe being ligatable to the 5' end of a first downstream probe in the presence of target to form a primary ligation product and the 3' end of a second upstream probe being

ligatable to the 5' end of a second downstream probe in the presence of primary ligation product to form a secondary ligation product; (b) reacting under hybridizing conditions said probes and sample suspected to contain said target nucleic acid; (c) ligating the upstream probe to the respective downstream probe using a thermostable ligase; (d) repeatedly denaturing the hybridized, ligated strands, reannealing additional probes and ligating them; and (c) detecting to what extent ligation products have formed; the improvement comprising performing said step b under conditions wherein the concentration of monovalent cation is greater than about 100 mM. Preferably the concentration of monovalent cation is between about 100 mM and 200 mM or at least about 180 mM. Generally, the monovalent cation is a metal, such as Na^+or K^+, or NR_3H^+, where R is independently selected from H or lower alkyl. KCl is a suitable supply of monovalent cation.

In a second aspect, the invention provides a method of detecting a small mutation as above, wherein the improvement comprises performing said step b under conditions wherein the initial temperature of mixing ranges from about 50-95 °C, followed by a lower temperature to enhance annealing. Preferred are ranges from about 60-85 °C, and from about 80-85 °C. This method, known as "hot start" may, but need not, be employed in conjunction with the high monovalent cation concentrations noted above.

In another aspect, the invention provides a method of detecting a small mutation as above, wherein the improvement comprises providing as at least one of said downstream probes, a mismatched probe having near its 5' end at least one base which is not complementary to the corresponding base in the target. This mismatched base is preferably within 5 bases from the 5' end and is also mismatched in the complementary probe so that the probes do not mismatch one another. This method may, but need not, be employed in conjunction with either or both of the high monovalent cation concentrations noted above and the "hot start" method.

In yet another aspect, the invention provides several distinct probe sets that are useful in LCR™ for detecting cystic fibrosis mutations designated ΔF_{508}, $G_{551}D$, $W_{1282}X$, $G_{542}X$, and the $J_{3.11}$ polymorphism.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 to 4 show the alignment of specific oligos with sections of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene. In each of these figures, Sequence ID Nos. are given in the left-most column (targets are not assigned a Sequence ID No. in these figures) and the numbering is that of Zielenski, et al. *Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene* , Genomics **10,** 214-228 (1991). In these figures, mutation changes in DNA are noted by placing the mutant base in parentheses just above or below the

normal base. Deletion mutations are indicated by asterisks. Underlined bases in oligos represent non-homologous tails added to increase the length and ease resolution of these amplification products from amplification products of the other allele.

Figure 1 shows the alignment of oligos 1-17 with the CFTR Gene ΔF_{508} mutation. This mutation involves a three base deletion (either TCT at DNA position 1652-54 or CTT at DNA position 1653-55, both of which leave a ΔF_{508} mutated sequence of ATTGGT in this region). These deletions cause the loss of a phenylalanine residue which occurs at position 508 of the normal CFTR protein.

Figure 2 shows the alignment of oligos 24-49 with the CFTR Gene $G_{551}D$ mutation. This mutation involves a G to A change at DNA position 1784, whereby GGT, which codes for glycine, becomes GAT, which codes for aspartic acid at protein position 551.

Figure 3 shows the alignment of oligos 18-23 with the CFTR Gene $W_{1282}X$ mutation. This mutation involves a G to A change at DNA position 3978, whereby TGG, which codes for tryptophan, becomes TGA, a stop codon, at protein position 1282.

Figure 4 shows the alignment of oligos 58-67 with the CFTR Gene $G_{542}X$ mutation. This mutation involves a G to T change at DNA position 1756, whereby GGA, which codes for glycine, becomes TGA, a stop codon, at protein position 542.

Figures 5, 6, 9 (a-f), 12 and 13 are autoradiograms of gels run in examples 7, 8, 11, 13, and 14, respectively. Detailed descriptions are provided in the respective examples.

Figures 7 (a-c), 8, 11 and 14 (a-b) are digitized photos of immunochromatographic strips run in examples 9, 10, 12 and 15, respectively. Detailed descriptions are provided in the respective examples.

Figure 10 shows the alignment of oligos 52-57 with the double stranded synthetic target identified as Sequence ID Nos. 50 and 51. Synthetic targets 50 and 51 were synthesized to represent the "antisense" allelic strands of a "normal" sequence containing C at position 23 (No. 50) and a "mutant" strand containing T instead (No. 51).

DETAILED DESCRIPTION

Mutations in DNA can take many forms. As compared to the DNA of a normal individual, mutated DNA may be of three general types. First, it may be missing one or more nucleotides. This is referred to as a "deletion". Second, it may contain one or more additional nucleotides. This is referred to as an "insertion" or "addition". Both deletions and insertions can cause "frame shift" translation errors if they occur in a coding region or "exon" of the gene. Such translation errors can cause the synthesis of proteins whose activity is reduced or totally absent. Finally, mutated DNA may contain

the same number of nucleotides, but may have a different nucleotide at one or more crucial position in the gene. This type of mutation, referred to as a "change" or "substitution", can also cause translational errors and poorly functioning proteins. The term "mutation" encompasses all three types described above.

A "small mutation" is a "mutation" as described above provided the number of affected nucleotides is not too large. For purposes of this invention, a mutation is "small" when the number of affected nucleotides is less than about 20% of the average size of the probes used to amplify and detect it. Thus, for a typical LCR™ probe set, the average size is about 15-25 nucleotides long. Thus, mutations of up to about 5 nucleotides are typically covered, although for longer probes, longer mutations would be considered "small". More usually, the mutation will only be about 15% of the average probe length, typically 3 or less bases. Of course, "point" mutations, in which a single nucleotide is deleted, inserted or changed, are clearly within the present invention and are probably the simplest to examine.

It is expected, however, that larger deletions and insertions can be suitably detected by the methods of the present invention, provided their ends are well defined and known. In the case of a large deletion, probe sets can be designed so that the common probe (*see infra*) hybridizes to the target on one side of the deletion segment; probes for the mutant allele are designed to hybridize to the target at the other end of the deletion segment; and probes for the normal allele are designed to hybridize to the deletion segment at the end adjacent the common probe set . Similarly, in the case of a large insertion, probe sets can be designed so that the common probe (*see infra*) hybridizes to the target on one side of the insertion segment; probes for the normal allele are designed to hybridize to the target at the other end of the insertion segment; and probes for the mutant allele are designed to hybridize to the inserted segment at the end adjacent the common probe set .

On the other hand, several types of large mutations present situations that are less than ideal for the present invention. For example, when many nucleotide changes are found within a relatively short region, the number of permutations of probe sets becomes impractical unless they can be examined as multiple cases of small mutations or unless nature provides (*e.g.* by natural selection) only a limited number of relevant polymorphisms that are well defined. Similarly, repeating mutations, such as tandem or dinucleotide repeats, would not be ideally suited for detection by these methods; nor would expansion mutations, such as are characteristic of fragile-X and myotonic dsytrophy.

A "target sequence" or "target nucleic acid" is a segment of nucleic acid (DNA or RNA). The segment may be from about 10 or 15 to several hundred nucleotides in length. For LCR™, a target segment is usually about 30 to about 60 nucleotides and the sequence is known. A target is "putative" if its presence is expected or anticipated,

or if it or a variation of it is expected or anticipated. For example, in multiplex LCR™ of an unknown homozygote to determine which of two mutually exclusive alleles is present, the sequences of both alleles are putative target sequences, even though only one is expected to be present. The possible alternatives for each allele are all putative targets.

As used in this application, a "multiplex" process refers to the carrying out of a method or process simultaneously and in the same reaction vessel on two or more different target sequences. Thus, multiplex LCR™ is the performance of LCR™ on a plurality of targets using at least one set of four probes for each putative target sequence (subject to the situation described below with regard to "common" probes). In like manner, the term "N-plex" LCR™, where N is a numeral, refers to LCR™ performed to amplify or detect one or more of N target sequences. Multiplex LCR™ is described in detail in the aforementioned U.S. application No.07/860,702, filed March 31, 1992.

While multiplex LCR™ is not essential to the present invention, a special case of duplex LCR™ is useful with the present invention. This special case of multiplex, which also can be viewed as "competitive" LCR™, occurs when information is desired simultaneously about both the mutant and the normal allele for a single mutation, for example, when heterozygote carrier status must be determined. In this case, probes designed to be specific for the normal allele essentially "compete" with probes designed to be specific for the mutant allele, since their target is the same except for the mutation. Certain advantages can be gained in this process because competition appears to enhance specificity, the prefectly matched probe being ligated more successfully than the imperfectly matched probe. Example 13 illustrates this principle and the need to optimize the balance of probe concentrations. Multiplex LCR™ is especially useful when more than one mutation locus must be examined to diagnose a particular disease or condition.

The Ligase Chain Reaction (LCR™) has been described in the prior art (See *e.g.* EP-A-320 308 and EP-A-439 182). Briefly, it is a process for amplifying a known target nucleic acid sequence using two primary probes (first and second, both of same sense) and two secondary probes (third and fourth, both of opposite sense with respect to primary probes). The first probe hybridizes to a first segment of the target strand and the second probe hybridizes to a second segment of the target strand, the first and second segments being contiguous so that the 3' hydroxyl end of an "upstream" probe abuts the 5' phosphate end of a "downstream" probe, and so that a ligase can covalently ligate the two probes into a fused primary ligation product.

In like manner, LCR™ employs upstream and downstream secondary probes. A third probe (downstream secondary) can hybridize to the first probe portion of the primary ligation product (*i.e.* that portion formed principally by the upstream primary probe) and a fourth probe (upstream secondary) can hybridize to the second probe

portion of the primary ligation product (*i.e.* the portion formed principally by the downstream primary probe) in a similar abutting fashion, such that the 5' end of the third probe (downstream secondary) can be ligated to the 3' end of the fourth (upstream secondary) probe to form a secondary ligation product. Of course, if the target is initially double stranded, the secondary probes can also hybridize to the target complement in the first instance.

Once the primary and secondary ligation products are separated from their respective complementary strands, they can hybridize with the secondary and primary probes, respectively. It is important to realize that the ligation products are functionally equivalent to either the target or its complement. By repeated cycles of hybridization, ligation and separation, amplification of the target sequence is achieved.

The step of "ligating" as recited above encompasses several known methods of joining two probes together. The preferred method is by the use of a thermostable ligase, especially a thermostable bacterial ligase, such as that of the *Thermus* genus. Ligases are discussed in EP-A-320 308 and in EP-A-373 962. Joining also encompasses the possible intermediate step of "correcting" a modified end as taught in EP-A-439 182. Correction includes the filling of a gap with an extending reagent (such as polymerase) as well as the cleaving of a blocking group (such as with endonuclease IV).

A reaction solution is typically prepared by collecting the sample from a patient and disrupting the cells to release the DNA. Detergents may be used, but other known methods of sample preparation are also included. Specific extraction buffer compositions are available from the literature, and from the examples. The DNA is rendered single-stranded by altering the stringency conditions, usually by heating.

It has unexpectedly been found that the use of high concentrations of monovalent cations enhances the present invention. While typical reaction buffers of the prior art have included the monovlaent cations NaCl or KCl at about 20 - 30 mM, the present invention employs up to 10 fold higher concentrations: for example, from about 100 to about 200 mM. Preferably, the monovalent cation is a metal ion such as K^+ or Na^+, or is an ammonium ion of the form NHR_3^+, where R is independently selected from H or lower alkyl. "Lower alkyl" in this application refers to monovalent straight or branched aliphatic radicals which may be derived from alkanes by the removal of one hydrogen, and have the general formula C_nH_{2n+1}; wherein n is from 1 to about 6. Examples of lower alkyl include CH_3-, CH_3CH_2-, $CH_3CH(CH_3)$-, and $CH_3(CH_2)_4$-.

Moreover, it has also been found that specificity can be improved by initiating the amplification reaction at a relatively high temperature; *i.e.* a temperature above about 50° C. Typically, the reaction is initiated when ligase is added to a mixture of denatured probes and target. When this mixing takes place at temperatures from 50-95°

C, the specificity is improved. Preferably, the reaction is started at 60-85°, more preferably 80-85° C. The reaction may also be started by supplying a necessary cofactor or other required component while the reaction mixture is at an elevated temperature.

To facilitate detection at least one probe of each probe set should bear a detectable label. Moreover, for multiplex LCR™ the detectable labels from each of the probe sets must all be differentiable, one from the other, either by signal differentiation or by spatial differentiation. As is described in more detail in U.S. application No.07/860,702, signal differentiation refers to the ability to distinguish targets in essentially the same location (i.e homogeneous assay) by virtue of differences in the signal (*e.g.* different fluorescent emission wavelengths or different colors). By contrast, spatial differentiation refers to the ability to distinguish targets based on the position or location of the signal. Spatial differentiation is also known as separation and may be accomplished by size, molecular weight, charge density, or magnetic or specific binding properties, and the like. Dot-blots, electrophoretic gels and immunochromatography are examples of spatial differentiation.

While a detectable label on at least one probe is important to the present invention, the nature of the label is not crucial. Isotopic labels can be detected by gel electrophoresis and autoradiography as in example 5. Hapten labels can be detected by solid phase separation and development, such as in the immunochromatography detection of example 6. Several labels, including hapten labels for capture and detection are described in the examples and in U.S. application No.07/860,702.

Probe Design

Multiplex LCR™ generally requires probes equalling four times the number of putative sequences. However, by careful probe design, the general rule of four probes for each putative target is modified in the present case, where the mutations are "small" and well defined. Two probes from one set (say, arbitrarily, the right two from the normal allele) will also serve as the two (right) probes for the mutant allele and only six probes will be required at each mutation to derive information about both alleles.

It will be noted that the common probes may be on the same side of the point of ligation with reference to a fixed point (*i.e.* they are an upstream probe and its complementary downstream probe). For example, left-side probes can serve as the common probes for CF mutations ΔF_{508} (oligos 1 and 2, Table 1 and fig. 1), $G_{551}D$ (oligos 24 and 25, Table 1 and fig. 2), and $W_{1282}X$ (oligos 18 and 19, Table 1 and fig. 3). In like manner, right-side probes can serve as common probes, *e.g.* for CF mutation $G_{542}X$ (oligos 62 and 63, Table 1 and fig. 4). "Right" and "left" side probes in this context refer to the complementary probes which are distal and proximal, respectively, of the point of ligation with reference to the origin of replication.

Alternatively, it will be noted that the common probes need not be those on the same side of the point of ligation (*i.e.* they need not be an upstream probe and its complementary downstream probe). Common probes can span the ligation junction, *i.e.* they may both be upstream or downstream probes on opposite strands. This is demonstrated by probes 14 and 15 (Figure 1) for CF mutation ΔF_{508} (Oligo 14 is a primary downstream probe on the right side and oligo 15 is a secondary downstream probe on the left side); and by probes 67 and 68 (Figure 4) for CF mutation $G_{542}X$ (Oligo 68 is a primary downstream probe on the right side and oligo 67 is a secondary downstream probe on the left side). In cases where common probes are used, it is possible to determine both alleles using only six distinct probes.

It can be seen that as a general rule, probes are designed so that a terminal nucleotide causes perfect complementarity with one allele (say, normal) and a mismatch viz-a-viz the mutant allele. In some cases, adequate specificity can be achieved by this mismatch alone, particularly when the mismatch results in a purine/purine or a pyrimidine/pyrimidine pairing. However, it has been found that the specificity of LCR™ is improved when one or more additional mismatches are deliberately introduced into the oligonucleotide probes, particularly near the 5' end of the downstream probes. "Near" in this context, means within about 5 bases from the 5' end. If the deliberate mismatch is too far from the end, it would not be expected to improve specificity.

Such an additional mismatch deliberately introduced into the 5' end of a downstream probe will necessarily be mismatched with respect to target in both alleles. However, it is preferred to modify the complementary upstream probe as well, so that the two probes are complementary at this additional site. See examples 11 and 12 for details. Although only one base is mismatched in the examples, in some cases it may be desirable to additionally mismatch more than one base in each probe.

It will also be noted from examination of Figures 1-3 that the outside ends of probes specific for the normal alleles have been "tailed" with sequences not necessarily homologous to the target, in these cases, *poly*(cytosine). The "outside ends" in this context refers to the probe ends which do not participate in the ligation reaction. This "tailing" serves to add length to the probe set specific for one or the other allele (it need not be the "normal" allele) so that the two ligation products can be differentiated on a gel when separated on the basis of length (or weight). This tailing should not be complementary to the target.

Such tailing may also be useful in a detection scheme. For example, the "tail" can be made complementary to a capture probe which might be immobilized on a membrane or other solid phase. Alternatively, the "tail" might be made complementary to a labeled detector probe, thus providing a way to identify the presence or not of the

amplification product. However, these tails are not essential to the invention and are provided merely to facilitate a more convenient method of detection.

This also serves to emphasize, however, that the exact length of the probes, *i.e.* the outside terminus, is not crucial to the invention. Probes that are somewhat longer, or somewhat shorter than the disclosed and claimed probes remain within the invention. However, as noted in application No. 07/860,702, when using multiplex LCR™, it is important to balance the melt temperatures among all the probes. Thus, the probe length becomes more critical in this situation.

It will be further noted that it may, in some cases, be appropriate to utilize combinations of the above techniques for improving specificity. For example, the combination of hot start and high salt concentrations may improve specificity beyond what either can do alone. Similarly, choice of probe design to introduce one or more additional mismatches could be used in combination with hot start or high salt. Alternatively, the methods described above may be used as alternatives. One method may alleviate or eliminate the need to use a second one. For example, choice of probe design to introduce one or more additional mismatches may alleviate or eliminate the need to use hot start or high salt. Such combinations and trade offs are also within the present invention.

One skilled in the art will readily recognize that the flexibility and "dynamic range" of various probe sets can be expanded by optimizing one or more of the above mentioned properties. Thus, the probe sets become more versatile and more robust, particularly when used in multiplex situations. An object of the invention is thus achieved.

EXAMPLES

The invention will now be described further by way of examples. The examples are illustrative of the invention and are not intended to limit it in any way. Unless otherwise indicated, probe and target sequences in the examples are written with the 5' end toward the left.

The following abbreviations are used in the examples and have the meaning indicated below:

BSA	bovine serum albumin
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
EDTA	a metal chelator, ethylenediamine tetraacetic acid
EPPS	a buffer comprising N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid)
oligos	oligonucleotides, generally oligo-2-deoxyribonucleotides.
NAD or NDA^+	nicotine adenine dinucleotide
TRIS	a buffer comprising *tris*(hydroxymethyl)aminomethane
TRITON	a detergent, O-(polyoxyethylene)-4-octylphenol

Mutations are described herein, in a manner generally accepted in the art. For example, ΔF_{508} specifies a three base deletion at DNA position 1653 which causes the loss of the phenylalanine which occurs at position 508 of the normal CFTR protein; $G_{551}D$ specifies a G to A change at DNA position 1784, causing replacement of the normally-occurring glycine at position 551 with aspartic acid; and $G_{542}X$ and $W_{1282}X$ specify truncation of the CFTR protein after positions 541 or 1281, respectively, because these so-called nonsense mutations (G to T at DNA position 1756 for $G_{542}X$, and G to A at DNA position 3978 for $W_{1282}X$) cause the gene to code for no amino acid at positions 551 or 1282, rather than the normally occurring glycine or tryptophan.

Example 1— Oligo synthesis

Oligonucleotides (oligos) were synthesized following standard protocols using automated synthesizers and nucleoside cyanoethylphosphoramidites (Applied Biosystems, Foster City, CA). For immunochromatographic detection, the probes were labeled at either the 3'- or 5'-end with a hapten as specified in Table 1. The labeling procedure followed standard solid-phase DNA synthesis methods, and used either commercially available haptens (Fluorescein-ON™ and Biotin-ON™, Clontech, Palo Alto, CA) or a dansyl phosphoramidite synthesized at Abbott Laboratories.

The sequences were selected based on the mutation to be detected and the method of LCR™. They were derived from the published sequence of the Cystic Fibrosis (CF) Transmembrane Conductance Regulator gene (J. Zielenski, R. Rozmahel, D. Bozon, B-S. Kerem, Z. Grzelczak, J. Riordan, J. Rommens, and L-C Tsui: *Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene* (1991) Genomics **10**, 214-228).

The specific sequences used are listed in Table 1. The manner in which oligos 1-17 align with the CFTR, in particular with the ΔF_{508} mutation, is shown in fig. 1. The manner in which oligos 24-33 align with the CFTR gene, in particular with the $G_{551}D$ mutation, is shown in fig. 2. The manner in which oligos 18-23 align with the CFTR gene, in particular with the $W_{1282}X$ mutation, is shown in fig. 3. The manner in which oligos 42-49 align with the CFTR gene, in particular with the $G_{542}X$ mutation, is shown in fig. 4.

TABLE 1
DNA SEQUENCES

SEQ ID No.	SEQUENCE
1.	5'-GCACCATTAA AGAAAATATC A-3'
2.	5'-GATATTTTCT TTAATGGTGC C-3'
3.	5'-CTTTGGTGTT TCCTATGATG CCCGGG-3'
4.	5'-GGGCCCATCA TAGGAAACAC CAAAGA-3'
5.	5'-TGGTGTTTCC TATGATGAAT-3'

6. 5′-TTCATCATAG GAAACACCAA-3′
7. 5′-TGATATTTTC TTTAATGGTG CC-3′
8. 5′-GCACCATTAA AGAAAATATC AT-3′
9. 5′-ATGATATTTT CTTTAATGGT GC-3′
10. 5′-GGGCCCCATC ATAGGAAACA CCAAAG-3′
11. 5′-ATTCATCATA GGAAACACCA-3′
12. 5′-dansyl-CACCATTAAA GAAAATATCA TCTT-3′
13. 5′-biotin-TATATTCATC ATAGGAAACA CCAAAG-3′
14. 5′-TGGTGTTTCC TATGATGAAT ATA-biotin-3′
15. 5′-ATGATATTTT CTTTAATGGT GCC-3′
16. 5′-fluorescein-GGCACCATTA AAGAAAATAT CAT-3′
17. 5′-biotin-CTATATTCAT CATAGGAAAC ACCA-3′

18. 5′-ATTCAATAAC TTTGCAACAG TG-3′
19. 5′-CACTGTTGCA AAGTTATTGA AT-3′
20. 5′-GAGGAAAGCC TTTGGAGTCC CCC-3′
21. 5′-CCCCCACTCC AAAGGCTTTC CTC-3′
22. 5′-AAGGAAAGCC TTTGGAGT-3′
23. 5′-ACTCCAAAGG CTTTCCTT-3′

24. 5′-TGGAATCACA CTGAGTGGAG-3′
25. 5′-CTCCACTCAG TGTGATTCCA-3′
26. 5′-GTCAACGAGC AAGAATTTCC CCCC-3′
27. 5′-CCCCCGAAAT TCTTGCTCGT TGAC-3′
28. 5′-ATCAACGAGC AAGAATTTC-3′
29. 5′-GAAATTCTTG CTCGTTGAT-3′
30. 5′-GGCAACGAGC AAGAATTTCC CCCC-3′
31. 5′-CCCCCGAAAT TCTTGCTCGT TGCC-3′
32. 5′-GTAAACGAGC AAGAATTTCC CCCC-3′
33. 5′-CCCCCGAAAT TCTTGCTCGT TTAC-3′
34. 5′-GTTAACGAGC AAGAATTTCC CCCC-3′
35. 5′-CCCCCGAAAT TCTTGCTCGT TAAC-3′
36. 5′-GTCCACGAGC AAGAATTTCC CCCC-3′
37. 5′-CCCCCGAAAT TCTTGCTCGT GGAC-3′
38. 5′-GTCACCGAGC AAGAATTTCC CCCC-3′
39. 5′-CCCCCGAAAT TCTTGCTCGG TGAC-3′
40. 5′-AGCAACGAGC AAGAATTTC-3′
41. 5′-GAAATTCTTG CTCGTTGCT-3′
42. 5′-ATAAACGAGC AAGAATTTC-3′

TABLE 1 (continued)
DNA SEQUENCES

43. 5′-GAAATTCTTG CTCGTTTAT-3′
44. 5′-ATTAACGAGC AAGAATTTC-3′
45. 5′-GAAATTCTTG CTCGTTAAT-3′
46. 5′-ATCCACGAGC AAGAATTTC-3′
47. 5′-GAAATTCTTG CTCGTGGAT-3′
48. 5′-ATCACCGAGC AAGAATTTC-3′
49. 5′-GAAATTCTTG CTCGGTGAT-3′
50. 5′-ATATTGTTGT GTCAGGACCA GCATTCCGGG AAAGGGGAGC AATAAGGTCA-3′
51. 5′-ATATTGTTGT GTCAGGACCA GCATTCCAGG AAAGGGGAGC AATAAGGTCA-3′
52. 5′-dansyl-ACCTTATTGC TCCCCTTTCC C-3′
53. 5′-GGAAAGGGGA GCAATAAGGT-3′
54. 5′-GAATGCTGGT CCTGACAC-biotin-3′
55. 5′-biotin-GTGTCAGGAC CAGCATTCC-3′
56. 5′-dansyl-ACCTTATTGC TCCCCTTTAC C-3′
57. 5′-GTAAAGGGGA GCAATAAGGT-3′

58. 5′-TGCAGAGAAA GACAATATAG TTCTTGGAGA AGGTGGAATC ACACTGAGTG G-3′
59. 5′-TGCAGAGAAA GACAATATAG TTCTTTGAGA AGGTGGAATC ACACTGAGTG G-3′
60. 5′-fluorescein-AAAGACAATA TAGTTCCTT-3′
61. 5′-AAGGAACTAT ATTGTCTTT-3′

62. 5'-GAGAAGGTGG AATCA-biotin-3'
63. 5'-biotin-TGATTCCACC TTCTC-3'
64. 5'-dansyl-AAAGACAATA TAGTTCGTG-3'
65. 5'-CACGAACTAT ATTGTCTTT-3'
66. 5'-dansyl-AAAGACAATA TAGTTCTTG-3'
67. 5'-AAGAACTATA TTGTCTTTC-3'
68. 5'-GAGAAGGTGG AATCAC-biotin-3'
69. 5'-biotin-TGATTCCACC TTCTCC-3'
70. 5'-fluorescein-AAAGACAATA TAGTTCTTT-3'
71. 5'-biotin-TGATTCCACC TTCTCA-3'

Example 2— Blunt LCR™

The LCR™ was performed using the so-called "blunt" strategy as described by Backman, *et al.* in European Patent Application 0 320 308 (1988). Reactions were performed in a total volume of 50 µL with the following final concentrations: 50 mM N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid)(EPPS) at pH 7.8, 10 mM $MgCl_2$, 10 mM NH_4Cl, 100 µM nicotine adenine dinucleotide (NAD), 100 µg/mL bovine serum albumin (BSA), and other concentrations as specified in subsequent examples. Ligation was accomplished using a heat-stable ligase isolated from *Thermus thermophilus*. DNA samples were obtained from the blood of affected cystic fibrosis (CF) patients, asymptomatic carriers, or normal human volunteers. The whole blood was obtained by venipuncture, and the DNA extracted using a DNA extractor (Applied Biosystems, Foster City, CA). The LCR™ process itself occurred in either a Tempcycler™ (Coy, Ann Arbor, MI) or a model 480 Thermal Cycler (Perkin-Elmer, Norwalk, CT) using temperature profiles specified in subsequent examples.

Example 3— Blunt LCR™ with rapid cycling

Blunt LCR™ similar to example 2 was also performed in a total volume of 15 µL with the following final concentrations: 20 mM *tris*(hydroxymethyl)aminomethane (TRIS) at pH 7.6, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 0.6 mM NAD, 0.1 % Triton X-100, and other concentrations as specified in subsequent examples. DNA samples were obtained as described in example 2. Alternatively, synthetic 51-base targets with sequence identical to the wild-type or mutant sequence were used. The LCR™ process itself occurred in a FTS-1 Thermal Sequencer™ (Corbett Research, Mortlake, NSW, Australia) using a temperature profile specified in subsequent examples.

Example 4— Gap LCR™

The LCR™ was performed using the so-called "single-gap" or "double-gap" strategy as described by Backman, *et al.* in European Patent Application 0 439 182 (1991). Reactions were performed in a total volume of 50 µL with the following final

concentrations: 50 mM EPPS pH 7.8, 20 mM potassium (added as KOH to adjust the pH of the buffer and as KCl to achieve 20 mM K^+), 30 mM $MgCl_2$ 100 µM NAD, 1 µM nucleoside triphosphate (dATP, dCTP, dGTP, or TTP as necessary to fill the particular gap), 0.5 unit/50 µL reaction DNA polymerase (Amplitaq®, Perkin-Elmer/Cetus, Emeryville, CA), and other concentrations as specified in subsequent examples. DNA samples were obtained as described in example 2. The LCR™ process itself occurred in either a Tempcycler™ (Coy, Ann Arbor, MI) or a model 480 Thermal Cycler (Perkin-Elmer, Norwalk, CT) using temperature profiles specified in subsequent examples.

Example 5— Detection of LCR™ by electrophoresis and autoradiography

For electrophoretic detection, one of the LCR™ probes was labeled at its 5'-end with ^{32}P following standard procedures (Sambrook, *et al.: Molecular cloning: a laboratory manual* (1989) Cold Spring Harbor Laboratory Press). Following LCR™, 10 µL portions of each reaction mixture were lyophilized and resuspended in 3 µL of 95% formamide, 20 mM EDTA with 0.05% bromophenol blue and 0.05% xylene cyanol, and loaded onto a 12% polyacrylamide gel containing 7 M urea. Following electrophoresis, the gels were exposed for autoradiography to Hyperfilm® (Amersham, Arlington Heights, IL).

Example 6— Detection of LCR™ by immunochromatography

Antisera to fluorescein and biotin were raised in rabbits against fluorescein-BSA or biotin-BSA. Antiserum against dansyl was a mouse monoclonal obtained from the University of Pennsylvania (S-T. Fan and F. Karush: Molecular Immunology (1984) 21, 1023-1029). These antisera were purified by passage through protein A Sepharose® or protein G Sepharose® (Pharmacia, Piscataway, NJ) and diluted in 0.1 M TRIS pH 7.8, 0.9% NaCl, 0.1% BSA, 1% sucrose, and a trace of phenol red. Portions (0.2 µL) of these diluted antisera were spotted onto 4 x 40 mm strips of nitrocellulose (AE 98, 5 µm, Schleicher and Schuell, Dassel, Germany).

Colloidal selenium was prepared following the procedure of D. A. Yost *et al.* (U. S. Patent 4,954,452). The colloid was diluted in water to achieve an optical density of 16 at 545 nm. To 1 mL of this suspension was added 1 µL of *anti*-biotin at 1 mg/mL and 60 µL of BSA at 100 mg/mL. This suspension was mixed on a vortex mixer for 1 minute, and was ready for use.

Alternatively, *anti*-biotin antiserum was conjugated to polystyrene uniformly-dyed blue latex particles (Bangs Laboratories, Carmal, IN). Particles (380 nm diameter) were diluted 1/25 in water to give 1 mL at 0.4% solids. and 10 µL of *anti*-biotin at 1

mg/mL was added. The suspension was mixed on a vortex mixer for 45 seconds, and 5 µL of 5% casein in 0.1 M TRIS pH 7.8 was added.

The LCR™ reaction mixtures were analyzed by Abbott TestPack Plus™ immunochromatography essentially following the protocol of European Patent Application EP-A-357-011. Briefly, 15 µL of the *anti-biotin* colloid (selenium or blue latex) was diluted with 14 µl buffer (0.1 M TRIS pH 7.8, 0.9% NaCl, 3% alkali-treated casein), and mixed with 1 µL LCR™ product. A nitrocellulose strip containing *anti*-fluorescein or *anti*-dansyl, or both, was introduced to the colloid suspension, and chromatography was allowed to proceed for 5 minutes. The strip was dried. The presence of a colored spot at the locus of antibody application indicated the presence of a specific LCR™ product. In some instances, the intensity of color was measured by digitizing an image of the strip and assigning a value based on the gray level determined in the digitizing process.

Example 7— CF ΔF_{508} mutation, gap-LCR™: electrophoretic detection

Extracted DNA from patients either homozygous for the CF ΔF_{508} mutation ($\Delta F_{508}/\Delta F_{508}$ DNA, fig. 5), from heterozygous carriers (NL/ΔF_{508} DNA, fig. 5), or from normal human controls (NL/NL, fig. 5) was amplified by single-gap and double-gap LCR™ using TTP as the fill base as described in example 4. For the double-gap method, oligos 1-6 (Table 1) were present at 20 nM each, and oligo 1 was labeled at the 5'-end with ^{32}P. The reaction mixture, without enzymes, was incubated at 98°C for 3 minutes, then cooled to 80°C. DNA ligase and DNA polymerase were added to the mixture at 80°C. The LCR™ then proceeded for 45 cycles of 85°C for 30 seconds and 45°C for 45 seconds in a Perkin-Elmer thermal cycler. The reaction products were analyzed by electrophoresis as described in example 5. Normal DNA yielded only the 49 bp product of the ligation of oligos 1-4 (NL/NL; lane 2, fig. 5). Homozygous CF ΔF_{508} DNA yielded only the 43 bp product of the ligation of oligos 1, 2, 5, and 6 ($\Delta F_{508}/\Delta F_{508}$; lane 3, fig. 5). DNA from heterozygous carriers amplified both products (NL/ΔF_{508}; lane 4, fig.). Amplification in the absence of target DNA produced insufficient product to detect (None; lane 1, fig. 5).

For the single-gap method, oligos 1, 3, 4, 5, 6, and 7 were present at 20 nM each, and oligo 1 was labeled at the 5'-end with ^{32}P. The same cycling protocol was used as for double-gap LCR™, with the exception that the annealing temperature was 46°C. Normal DNA yielded only the 49 bp product of the ligation of oligos 1, 3, 4, and 7 (NL/NL; lane 6, fig. 5). Homozygous CF ΔF_{508} DNA yielded only the 43 bp product of the ligation of oligos 1, 5, 6, and 7 ($\Delta F_{508}/\Delta F_{508}$; lane 7, fig. 5). DNA from heterozygous carriers amplified both products (NL/ΔF_{508}; lane 8, fig. 5). Amplification in the absence of target DNA again produced very little, if any, product (None; lane 5, fig. 5).

Example 8— Effect of salt concentration on CF ΔF_{508} mutation, blunt LCR™: electrophoretic detection

Extracted DNA from patients either homozygous or heterozygous for the CF ΔF_{508} mutation was amplified by blunt LCR™ as described in example 2. Oligos 3, 8, 9, and 10 (Table 1) were used at 10 nM each, and oligos 5 and 11 were present at 15 nM. Oligo 8 was labeled at the 5'-end with ^{32}P. The reaction mixture, without enzyme, was incubated at 98°C for 3 minutes, then cooled to 80°C. DNA ligase was added to the mixture at 80°C. The LCR™ then proceeded for 42 cycles of 85°C for 30 seconds and 49°C for 45 seconds in a Perkin-Elmer thermal cycler. The reaction products were analyzed by electrophoresis as described in example 5. Both the 48 bp product of the ligation of oligos 3, 8, 9, and 10 and the 42 bp product of oligos 5, 8, 9, and 11 were amplified from normal DNA (NL/NL), homozygous DNA ($\Delta F_{508}/\Delta F_{508}$), and heterozygous DNA (NL/ΔF_{508}), and even in the absence of any DNA target (None) when the potassium (KCl) concentration was 20 mM or 100 mM (lanes 1-8, fig. 6). When the KCl concentration was 180 mM, then the 48 bp product was produced from normal DNA (lane 10, fig. 6) and the 42 bp product from homozygous DNA (lane 12, fig. 6). At 180 mM KCl, amplification of 48 bp product from homozygous DNA and 42 bp product from normal DNA were both greatly reduced. Both products were produced from heterozygous DNA (lane 12, fig. 6), but no detectable product appeared in the absence of DNA target (lane 9, fig. 6).

Example 9— Effect of salt concentration on the CF ΔF_{508} mutation, blunt-LCR™: immunochromatographic detection

DNA was extracted and amplified as described in example 7, except that the buffer used was that used for gap LCR™ (example 4). Oligos 12 and 13 were present at 27 nM and oligos 14-17 at 13.6 nM. The reaction mixture, without enzyme, was incubated at 100°C for 3 minutes, then cooled to 85°C. DNA ligase and 0.5 unit DNA polymerase were added to the mixture at 85°C (subsequent studies showed that the DNA polymerase was not necessary— only the detergent, which in this case was TWEEN-20). The LCR™ then proceeded for 42 cycles of 85°C for 1 minute and 55°C for 1 minute in a Perkin-Elmer thermal cycler. The reaction products were analyzed by immunochromatography as described in example 6. When the concentration of KCl was 80 mM or 100 mM, both the ligation product of oligos 12-15 containing dansyl at one end and biotin at the other, and the ligation product of oligos 13-17 containing fluorescein at one end and biotin at the other were amplified from homozygous (ΔF_{508}), heterozygous (het), and normal (W) DNA (figs. 7a and 7b). When the KCl concentration was 120 mM, then normal DNA amplified the dansyl-biotin product (W; strips 3 and 6, fig. 7c), and homozygous DNA amplified the fluorescein-biotin product

(ΔF_{508}; strips 1 and 4, fig. 7c). Amplificatiion of dansyl-biotin product by homozygous DNA and fluorescein-biotin product by normal DNA were below the detection limits of the system. Heterozygous DNA amplified both products (het; strips 2 and 5, fig. 7c).

Example 10— Effect of salt concentration on CF $G_{542}X$ mutation, blunt LCR™

Synthetic 51-base targets homologous for the normal CFTR gene (oligo 58, "N" in fig. 8) or for the $G_{542}X$ mutation (oligo 59, "M" in fig. 8) were amplified by blunt LCR™ with rapid cycling as described in example 3. Oligos 60-63 were present at 22 nM, 55 nM, or 110 nM each, and the synthetic targets were present at 1×10^6 copies each. The KCl concentration was 75 mM, 100 mM, or 125 mM, as specified in fig. 8 and table 2. The reaction mixture, with enzyme, was assembled at 0°C and transfered to the Corbett thermal sequencer. The LCR™ proceeded for 30, 35, or 40 cycles of 85°C for 5 seconds and 45°C for 5 seconds. A conplete cycle took approximately 55 seconds. The reaction products were analyzed by immunochromatography and quantitated by digital imaging as described in example 6.

The particular combinations of oligo concentration, KCl concentration, and extent of LCR™, along with the results achieved, are specified in table 2. In general, under the conditions used, 22 nM oligo was insufficient to obtain signal significantly above background, even after 40 cycles of LCR™ (conditions 4, 5, and 8, Table 2, below). On the contrary, 110 nM oligo was sufficient to obtain strong signal even after only 30 cycles (conditions 2, 6, 7, and 10, Table 2). The effect of increasing salt concentration was to suppress the signal from normal target (which has a mismatch with oligos 60 and 61 at the point of ligation) without affecting the signal from mutant target. The net result was to increase the discrimination between the normal and mutant targets (conditions 2 and 10, Table 2). In fact, there was no discrimination between these targets under any of the conditions at 75 mM KCl (conditions 9-11, Table 2).

TABLE 2
Reaction conditions used, and Results obtained, for experiment 10
CF $G_{542}X$ mutation

Condition (run number, figure 8)	number of LCR™ cycles	concentration of KCl (mM)	concentration of oligos (nM)	optical density with normal target	optical density with mutant target
1	30	125	55	16	18
2	35	125	110	16	77
3	40	125	55	16	27
4	35	125	22	15	20
5	30	100	22	14	20
6	30	100	110	13	58
7	40	100	110	111	86
8	40	100	22	17	18
9	30	75	55	20	18
10	35	75	110	60	58
11	40	75	55	31	26
12	35	100	55	24±9	25±6

Example 11— Effect of probe design on $G_{551}D$ mutation

Extracted DNA from patients heterozygous for the CF $G_{551}D$ mutation (HET DNA, figs. 9) or from normal human controls (N DNA, figs. 9) was amplified by blunt LCR™ as described in example 2. Oligos 24, 25, 26, 27, and 30-38 were present at 10 nM each and oligos 28, 29, and 40-49 at 8 nM each. Oligo 24 was labeled at the 5'-end with ^{32}P. The reaction mixture, without enzyme, was incubated at 98°C for 3 minutes, then cooled to 80°C. DNA ligase was added to the mixture at 80°C. The LCR™ then proceeded for 30 cycles of 85°C for 30 seconds and 49°C for 45 seconds in a Perkin-Elmer thermal cycler. The reaction products were analyzed by electrophoresis as described in example 5.

For analysis with homologous probes, the LCR™ was performed with "normal" oligos 24-27 (oligo N, fig. 9, producing a 44 bp product) or "mutant" oligos 24, 25, 28, and 29 (oligo M, fig. 9, producing a 39 bp product). Amplified product was produced by both sets of oligos from any of the human DNA targets (lanes 3-6,

fig. 9a) although little, if any, amplification was seen in the absence of target DNA (lanes 1-2, fig. 9a).

For analysis with probes containing as additional mismatch, the LCR™ was performed with the oligo sets as specified in Table 3, below. With these oligos, amplification occurred predominantly when the probes matched the target exactly at the point of ligation, *i.e.* "normal" oligos amplified control and heterozygous G551D DNA (lanes 3 and 5, figs. 9b, 9c, 9d, 9e, and 9f), and "mutant" oligos amplified only the heterozygous G551D DNA (lanes 4 and 6, figs. 9b, 9c, 9d, 9e, and 9f). Little amplification occurred in the absence of human DNA target (lanes 1 and 2, figs. 9b, 9c, 9d, 9e, and 9f). This pattern persisted, whether the additional mismatch was 1, 2, 3, or 4 bases removed from the point of ligation (see CF G551D oligo alignments, fig. 2).

TABLE 3
Oligo sets used for probe design experiment 11
CF G551D mutation

oligos (Table 1)	designation	Position of additional mismatch in 5' end (number of bases away from ligation point)	amplified size	reference figure
24, 25, 30, 31	normal (N)	1	44 bp	9b
24, 25, 40, 41	mutant (M)	1	39 bp	9b
24, 25, 32, 33	normal (N)	2	44 bp	9c
24, 25, 42, 43	mutant (M)	2	39 bp	9c
24, 25, 34, 35	normal (N)	2	44 bp	9d
24, 25, 44, 45	mutant (M)	2	39 bp	9d
24, 25, 36, 37	normal (N)	3	44 bp	9e
24, 25, 46, 47	mutant (M)	3	39 bp	9e
24, 25, 38, 39	normal (N)	4	44 bp	9f
24, 25, 48, 49	mutant (M)	4	39 bp	9f

Example 12— Effect of probe design on the CF J3.11 polymorphism

The CF $J_{3.11}$ mutation is a polymorphism loosely linked to cystic fibrosis, but removed from the CFTR gene by several hundred bp. It is a point mutation; I. Bartels, et al, *Am. J. Human Genetics*, 38: 280-7 (1986). Synthetic 50-base targets homologous for allele 1 or allele 2 of the CF $J_{3.11}$ polymorphism (oligos 50 and 51,

respectively) were amplified by gap LCR™ as described in example 4, using dGTP and dATP as the fill bases. Potassium as KCl was present at 100 mM. Oligos 52-57, where used, were present at 13.6 nM. The reaction mixture, without enzyme, was incubated at 100°C for 3 minutes, then cooled to 85°C. DNA ligase was added to the mixture at 85°C. The LCR™ then proceeded for 42 cycles of 85°C for 30 seconds and 45°C for 30 seconds in a Perkin-Elmer thermal cycler. The reaction products were analyzed by immunochromatography on strips spotted with *anti*-dansyl antibody as described in example 6.

The alignment of oligos 52-57 with the synthetic targets 50 and 51 is shown in fig. 10.

For analysis with homologous oligos, the LCR™ was performed with oligos 52-55. These oligos have perfect sequence homology with target 50, but have a mismatch with target 51 at the point of ligation. Nevertheless, these oligos amplified both targets (strips 1 and 2, fig. 11). A second analyses used oligos 53-56. In this set, one oligo (53) had perfect sequence homology with target 50, while its complement (56) had one mismatch. These oligos had 1 and 2 mismatches, respectively, with target 51. These oligos amplified neither target (strips 3 and 4, fig. 11). A third analysis used oligos 54-57, in which oligos 56 and 57 had 1 mismatch with respect target 50 and 2 mismatches with target 51, but *no* mismatches with respect to each other. These oligos amplified target 50 (strip 5) where probe and target share complete homology at the point of ligation, but target 51, which has a mismatch at the point of ligation, amplified very little (strip 6, fig. 11).

Example 13— Effect of probe balance on CF $W_{1282}X$ mutation

Extracted DNA from patients homozygous for the CF $W_{1282}X$ mutation ($W_{1282}X/W_{1282}X$ DNA, fig. 12), heterozygous for the $W_{1282}X$ mutation ($NL/W_{1282}X$ DNA, fig. 12) or from normal human controls (NL/NL DNA, fig. 12) was amplified by blunt LCR™ as described in example 2. Oligos 18-21 were present at 10 nM each and oligos 22 and 23 were present at 15, 16, 17, or 18 nM as indicated. Oligo 18 was labeled at the 5'-end with ^{32}P. The reaction mixture, without enzyme, was incubated at 98°C for 3 minutes, then cooled to 80°C. DNA ligase was added to the mixture at 80°C. The LCR™ then proceeded for 42 cycles of 85°C for 30 seconds and 50°C for 45 seconds in a Perkin-Elmer thermal cycler. The reaction products were analyzed by electrophoresis as described in example 5.

For the purposes of this example, oligos were classified as "normal" if they were completely homologous with the normal CFTR gene sequence (oligos 20 and 21, "N" in fig. 12), "mutant" if they were completely homologous with the sequence of the CF $W_{1282}X$ mutation (oligos 22 and 23, "M" in fig. 12), or "common" if they were completely homologous to either sequence (oligos 18 and 19, "C" in fig. 12).

Amplified product was produced in insufficient quantity to detect above background from control reactions containing no human DNA (lanes 1, 5, 9, and 13; fig. 12). The combination of normal (N) and common (C) oligos produced a 45 bp product from normal DNA (NL/NL, lanes 2, 6, 10, and 14; fig. 12) and from heterozygous DNA (NL/$W_{1282}X$, lanes 4, 8, 12, and 16; fig. 12), but little or no product from homozygous DNA ($W_{1282}X/W_{1282}X$, lanes 3, 7, 11, and 15; fig. 12). The combination of mutant (M) and common (C) oligos produced a 40 bp product from homozygous DNA ($W_{1282}X/W_{1282}X$, lanes 3, 7, 11, and 15; fig. 12) and from heterozygous DNA (NL/$W_{1282}X$, lanes 4, 8, 12, and 16; fig. 12), but little or no product from normal DNA (NL/NL, lanes 2, 6, 10, and 14; fig. 12).

The amount of amplified product produced, indicated by the relative intensities of the bands in fig. 12, depended very critically on the relative concentration of the oligos present. When the mutant oligos (M) were present at 15 nM, the spot from the 45 bp "normal" product was much more intense that that from the 40 bp "mutant" product (lanes 1-4, fig. 12). As the concentration of mutant oligos increased to 16, 17, and 18 nM, the spot from the 40 bp product increased in intensity, whereas the spot from the 45 bp product decreased in intensity (lanes 5-8, 9-12, and 13-16; fig. 12). It is important to note that very small changes in oligo concentration (as little as 5%) effected a major change in the relative amounts of the different amplified materials produced.

Example 14— Effect of hot start on CF $G_{551}D$ mutation

DNA was extracted and amplified as described in example 11 using oligos 24-29 at the concentrations stated in example 11. Oligo 24 was labeled at the 5'-end with ^{32}P. For LCR™ with "hot start" the reaction mixture, without enzyme, was incubated at 98°C for 3 minutes, then cooled to 40°C, 50°C, 60°C, or 80°C; at which temperature DNA ligase was added. For LCR™ without "hot start" the reaction mixture, without enzyme, was incubated at 98°C for 3 minutes, then cooled to room temperature. DNA ligase was added to the mixture at room temperature. Under both conditions, the LCR™ then proceeded for 34 cycles of 85°C for 30 seconds and 49°C for 45 seconds in a Perkin-Elmer thermal cycler. The reaction products were analyzed by electrophoresis as described in example 5.

Without hot start, or with hot start at 40°C, amplified product was produced in the presence of normal DNA (N, fig. 13) or heterozygous DNA (HET, fig. 13) with either normal oligos (oligos 24-27, oligo N of fig. 13, producing a 44 bp product; lanes 3, 5, 9, and 11) or mutant oligos (oligos 24, 25, 28, and 29; oligo M of fig. 13, producing a 39 bp product, lanes 4, 6, 10, and 12). Mutant oligos also produced an amplified product in the absence of human DNA at these temperatures (lanes 2 and 8, fig. 13).

With hot start at 50°C, 60°C, or 80°C, mutant oligos amplified only the heterozygous CF G551D DNA (HET, lanes 18, 24, and 30, fig. 13), and neither the normal human DNA (lanes 16, 22, and 28) nor the control without human DNA (lanes 14, 20, and 26). Similarly, normal oligos under hot start at these temperatures amplified normal human DNA (lanes 15, 21, and 27) and heterozygous CF G551D DNA (lanes 17, 23, and 29), but not the control without human DNA (lanes 13, 19, and 25).

Example 15— Effect of hot start on CF G542X mutation

Synthetic 51-base targets homologous for the normal CFTR gene (oligo 58, "N" in figs. 14) or for the G542X mutation (oligo 59, "M" in figs. 14) were amplified by blunt LCR™ with rapid cycling as described in example 3.

For amplification without hot start, oligos 66-69 were present at the concentrations stated in table 4. The reaction mixtures, complete with target, oligos, and enzyme, were assembled at room temperature and transferred to the Corbett thermal sequencer. The LCR™ proceeded for the number of cycles stated in table 4, each cycle comprising 85°C for 5 seconds and the lower temperature stated in table 4 for 5 seconds. The reaction products were analyzed by immunochromatography and quantitated by digital imaging as described in example 6.

For amplification with hot start, oligos 66-69 were present at 92 nM each. The reaction mixtures were prepared separately, with 8 µL of DNA ligase (1700 units) being introduced to the reaction capillary first, followed by an air space of 2.5 µL, and finally 10 µL of reaction mix containing oligos and target. The capillaries were placed in the Corbett thermal sequencer, heated to 85°C for 30 seconds, and the liquids mixed by compressing the contents of the capillary. This was easily accomplished by briefly pushing down on the plunger of the capillary syringe. The LCR™ proceeded for 40 cycles of 85°C for 5 seconds and 57°C for 5 seconds. The reaction products were analyzed by immunochromatography as described in example 6.

It can be seen from the data of fig. 14a and table 4 that there are no conditions under which significant signal resulted from the amplification of "normal" target without amplification of "mutant" target as well. Condition 2 (35 cycles, 53°C lower temperature, 33 nM oligos) gave a weak "normal" signal with very little "mutant" signal, but all conditions which yielded strong "normal" signal gave concomitant signal from the mutant target as well. In contrast, hot start yielded a strong signal from "normal" target with no appreciable signal from mutant target (fig. 14b).

TABLE 4
Reaction conditions used, and Results obtained, for experiment 15
CF $G_{542}X$ mutation

Condition (run number, figure 14)	number of LCR™ cycles	lower temperature (°C)	concentration of oligos (nM)	optical density with normal target	optical density with mutant target
1	32	53	66	64	61
2	35	53	33	26	9
3	35	53	133	83	82
4	38	53	66	75	76
5	32	55	33	6	7
6	32	55	133	83	75
7	38	55	133	93	85
8	38	55	33	15	12
9	35	57	133	69	34
10	35	57	33	8	7
11	38	57	66	57	19
12	32	57	66	6	7
13	35	55	66	22±4	43±11

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ABBOTT LABORATORIES and
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WANG-TING HSIEH
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(ii) TITLE OF INVENTION: LIGASE CHAIN REACTION METHOD FOR DETECTING SMALL MUTATIONS

(iii) NUMBER OF SEQUENCES: 75

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(A) MEDIUM TYPE: Floppy diskette
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Wordperfect

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCACCATTAA AGAAAATATC A 21

(3) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATATTTTCT TTAATGGTGC C 21

(4) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTTTGGTGTT TCCTATGATG CCCGGG 26

(5) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGGCCCATCA TAGGAAACAC CAAAGA 26

(6) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGGTGTTTCC TATGATGAAT 20

(7) INFORMATION FOR SEQ ID NO: 6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTCATCATAG GAAACACCAA 20

(8) INFORMATION FOR SEQ ID NO: 7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TGATATTTTC TTTAATGGTG CC 22

(9) INFORMATION FOR SEQ ID NO: 8:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCACCATTAA AGAAAATATC AT 22

(10) INFORMATION FOR SEQ ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATGATATTTT CTTTAATGGT GC 22

(11) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGGCCCCATC ATAGGAAACA CCAAAG 26

(12) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATTCATCATA GGAAACACCA 20

(13) INFORMATION FOR SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CACCATTAAA GAAAATATCA TCTT 24

(14) INFORMATION FOR SEQ ID NO: 13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TATATTCATC ATAGGAAACA CCAAAG 26

(15) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGGTGTTTCC TATGATGAAT ATA 23

(16) INFORMATION FOR SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ATGATATTTT CTTTAATGGT GCC 23

(17) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGCACCATTA AAGAAAATAT CAT 23

(18) INFORMATION FOR SEQ ID NO: 17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CTATATTCAT CATAGGAAAC ACCA 24

(19) INFORMATION FOR SEQ ID NO: 18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

ATTCAATAAC TTTGCAACAG TG 22

(20) INFORMATION FOR SEQ ID NO: 19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CACTGTTGCA AAGTTATTGA AT 22

(21) INFORMATION FOR SEQ ID NO: 20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GAGGAAAGCC TTTGGAGTCC CCC 23

(22) INFORMATION FOR SEQ ID NO: 21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CCCCCACTCC AAAGGCTTTC CTC 23

(23) INFORMATION FOR SEQ ID NO: 22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AAGGAAAGCC TTTGGAGT 18

(24) INFORMATION FOR SEQ ID NO: 23:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

ACTCCAAAGG CTTTCCTT 18

(25) INFORMATION FOR SEQ ID NO: 24:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TGGAATCACA CTGAGTGGAG 20

(26) INFORMATION FOR SEQ ID NO: 25:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CTCCACTCAG TGTGATTCCA 20

(27) INFORMATION FOR SEQ ID NO: 26:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTCAACGAGC AAGAATTTCC CCCC 24

(28) INFORMATION FOR SEQ ID NO: 27:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CCCCCGAAAT TCTTGCTCGT TGAC 24

(29) INFORMATION FOR SEQ ID NO: 28:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

ATCAACGAGC AAGAATTTC 19

(30) INFORMATION FOR SEQ ID NO: 29:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GAAATTCTTG CTCGTTGAT 19

(31) INFORMATION FOR SEQ ID NO: 30:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GGCAACGAGC AAGAATTTCC CCCC 24

(32) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CCCCCGAAAT TCTTGCTCGT TGCC 24

(33) INFORMATION FOR SEQ ID NO: 32:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GTAAACGAGC AAGAATTTCC CCCC 24

(34) INFORMATION FOR SEQ ID NO: 33:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CCCCCGAAAT TCTTGCTCGT TTAC 24

(35) INFORMATION FOR SEQ ID NO: 34:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GTTAACGAGC AAGAATTTCC CCCC 24

(36) INFORMATION FOR SEQ ID NO: 35:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CCCCCGAAAT TCTTGCTCGT TAAC 24

(37) INFORMATION FOR SEQ ID NO: 36:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GTCCACGAGC AAGAATTTCC CCCC 24

(38) INFORMATION FOR SEQ ID NO: 37:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CCCCCGAAAT TCTTGCTCGT GGAC 24

(39) INFORMATION FOR SEQ ID NO: 38:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GTCACCGAGC AAGAATTTCC CCCC 24

(40) INFORMATION FOR SEQ ID NO: 39:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

CCCCCGAAAT TCTTGCTCGG TGAC 24

(41) INFORMATION FOR SEQ ID NO: 40:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

AGCAACGAGC AAGAATTTC 19

(42) INFORMATION FOR SEQ ID NO: 41:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

GAAATTCTTG CTCGTTGCT 19

(43) INFORMATION FOR SEQ ID NO: 42:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

ATAAACGAGC AAGAATTTC 19

(44) INFORMATION FOR SEQ ID NO: 43:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

GAAATTCTTG CTCGTTTAT 19

(45) INFORMATION FOR SEQ ID NO: 44:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

ATTAACGAGC AAGAATTTC 19

(46) INFORMATION FOR SEQ ID NO: 45:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

GAAATTCTTG CTCGTTAAT 19

(47) INFORMATION FOR SEQ ID NO: 46:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

ATCCACGAGC AAGAATTTC 19

(48) INFORMATION FOR SEQ ID NO: 47:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

GAAATTCTTG CTCGTGGAT 19

(49) INFORMATION FOR SEQ ID NO: 48:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

ATCACCGAGC AAGAATTTC 19

(50) INFORMATION FOR SEQ ID NO: 49:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

GAAATTCTTG CTCGGTGAT 19

(51) INFORMATION FOR SEQ ID NO: 50:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

ATATTGTTGT GTCAGGACCA GCATTCCGGG AAAGGGGAGC AATAAGGTCA 50

(52) INFORMATION FOR SEQ ID NO: 51:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

ATATTGTTGT GTCAGGACCA GCATTCCAGG AAAGGGGAGC AATAAGGTCA 50

(53) INFORMATION FOR SEQ ID NO: 52:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

ACCTTATTGC TCCCCTTTCC C 21

(54) INFORMATION FOR SEQ ID NO: 53:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

GGAAAGGGGA GCAATAAGGT 20

(55) INFORMATION FOR SEQ ID NO: 54:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

GAATGCTGGT CCTGACAC 18

(56) INFORMATION FOR SEQ ID NO: 55:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

GTGTCAGGAC CAGCATTCC 19

(57) INFORMATION FOR SEQ ID NO: 56:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

ACCTTATTGC TCCCCTTTAC C 21

(58) INFORMATION FOR SEQ ID NO: 57:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

GTAAAGGGGA GCAATAAGGT 20

(59) INFORMATION FOR SEQ ID NO: 58:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

TGCAGAGAAA GACAATATAG TTCTTGGAGA AGGTGGAATC ACACTGAGTG G 51

(60) INFORMATION FOR SEQ ID NO: 59:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

TGCAGAGAAA GACAATATAG TTCTTTGAGA AGGTGGAATC ACACTGAGTG G 51

(61) INFORMATION FOR SEQ ID NO: 60:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

AAAGACAATA TAGTTCCTT 19

(62) INFORMATION FOR SEQ ID NO: 61:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

AAGGAACTAT ATTGTCTTT 19

(63) INFORMATION FOR SEQ ID NO: 62:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

GAGAAGGTGG AATCA 15

(64) INFORMATION FOR SEQ ID NO: 63:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

TGATTCCACC TTCTC 15

(65) INFORMATION FOR SEQ ID NO: 64:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

AAAGACAATA TAGTTCGT 18

(66) INFORMATION FOR SEQ ID NO: 65:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

CACGAACTAT ATTGTCTTT 19

(67) INFORMATION FOR SEQ ID NO: 66:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

AAAGACAATA TAGTTCTTG 19

(68) INFORMATION FOR SEQ ID NO: 67:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

AAGAACTATA TTGTCTTTC 19

(69) INFORMATION FOR SEQ ID NO: 68:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

GAGAAGGTGG AATCAC 16

(70) INFORMATION FOR SEQ ID NO: 69:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

TGATTCCACC TTCTCC 16

(71) INFORMATION FOR SEQ ID NO: 70:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

AAAGACAATA TAGTTCTTT 19

(72) INFORMATION FOR SEQ ID NO: 71:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

TGATTCCACC TTCTCA 16

(73) INFORMATION FOR SEQ ID NO: 72:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

TTTTCCTGGA TTATGCCTGG CACCATTAAA GAAAATATCA TCTTTGGTGT TCCTATGAT 60
GAATATAGAT ACAGAAGCG 89

(74) INFORMATION FOR SEQ ID NO: 73:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

ATATAGTTCT TGGAGAAGGT GGAATCACAC TGAGTGGAGG TCAACGAGCA AGAATTTCTT 60
TAGCAAGGTG AATAACT 87

(75) INFORMATION FOR SEQ ID NO: 74:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

ATCCAGATCG ATGGTGTGTC TTGGGATTCA ATAACTTTGC AACAGTGGAG GAAAGCCTTT 60
GGAGTGATAC CACAG 85

(76) INFORMATION FOR SEQ ID NO: 75:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

ATCCAGATCG ATGGTGTGTC TTGGGATTCA ATAACTTTGC AACAGTGGAG GAAAGCCTTT 60
GGAGTGATAC CACAG 85

What is claimed is:

1. In a method of detecting a small mutation in a target nucleic acid the sequence of which is known, said method including: (a) providing an excess of at least two sets of two probes, the 3' end of a first upstream probe being ligatable to the 5' end of a first downstream probe in the presence of target to form a primary ligation product and the 3' end of a second upstream probe being ligatable to the 5' end of a second downstream probe in the presence of primary ligation product to form a secondary ligation product; (b) reacting under hybridizing conditions said probes and sample suspected to contain said target nucleic acid; (c) ligating the upstream probe to the respective downstream probe using a thermostable ligase; (d) repeatedly denaturing the hybridized, ligated strands, reannealing additional probes and ligating them; and (c) detecting to what extent ligation products have formed;

the improvement comprising:

performing said step b under conditions wherein the concentration of monovalent cation is greater than about 100 mM.

2. The method of claim 1, wherein said concentration is at least about 180 mM.

3. The method of claim 1, wherein said monovalent cation is selected from the group consisting of Na^+, K^+ and NR_3H^+, where R is independently selected from H or lower alkyl.

4. The method of claim 1, wherein said probes are selected such that the 3' end of the upstream probe directly abuts the 5' end of the downstream probe when said probes are aligned on their respective targets.

5. The method of claim 1, wherein said probes are selected such that a gap of 1 or more bases exists between the 3' end of the upstream probe and the 5' end of the downstream probe when said probes are aligned on their respective targets, and further comprising the step of filling said gap to render the 3' end of the upstream probe directly abutting the 5' end of the downstream probe.

6. In a method of detecting a small mutation in a target nucleic acid the sequence of which is known, said method including: (a) providing an excess of at least two sets of two probes, the 3' end of a first upstream probe being ligatable to the 5' end of a first downstream probe in the presence of target to form a primary ligation product and the 3' end of a second upstream probe being ligatable to the 5' end of a second downstream probe in the presence of primary ligation product to form a secondary ligation product; (b) reacting under hybridizing conditions said probes and sample suspected to contain said target nucleic acid; (c) ligating the upstream probe to the respective downstream probe; (d) repeatedly denaturing the hybridized, ligated strands, reannealing additional probes and ligating them; and (c) detecting to what extent ligation products have formed;

the improvement comprising:

performing said step b under conditions wherein the initial temperature of mixing ranges from about 50-95 °C, followed by a lower temperature to enhance annealing.

7. The method of claim 6, wherein the initial temperature of mixing ranges from about 80-85 °C.

8. The method of claim 6, wherein said probes are selected such that the 3' end of the upstream probe directly abuts the 5' end of the downstream probe when said probes are aligned on their respective targets.

9. The method of claim 6, wherein said probes are selected such that a gap of 1 or more bases exists between the 3' end of the upstream probe and the 5' end of the downstream probe when said probes are aligned on their respective targets, and further comprising the step of filling said gap to render the 3' end of the upstream probe directly abutting the 5' end of the downstream probe.

10. The method of claim 6, further comprising performing said step b under conditions wherein the concentration of monovalent cation is is greater than about 100 mM.

11. The method of claim 10, wherein said monovalent cation is selected from the group consisting of Na^+, K^+ and NR_3H^+, where R is independently selected from H or lower alkyl.

12. In a method of detecting a small mutation in a target nucleic acid the sequence of which is known, said method including: (a) providing an excess of at least two sets of two probes, the 3' end of a first upstream probe being ligatable to the 5' end of a first downstream probe in the presence of target to form a primary ligation product and the 3' end of a second upstream probe being ligatable to the 5' end of a second downstream probe in the presence of primary ligation product to form a secondary ligation product; (b) reacting under hybridizing conditions said probes and sample suspected to contain said target nucleic acid; (c) ligating the upstream probe to the respective downstream probe; (d) repeatedly denaturing the hybridized, ligated strands, reannealing additional probes and ligating them; and (c) detecting to what extent ligation products have formed;

the improvement comprising:

providing as at least one of said downstream probes, a mismatched probe having near its 5' end at least one base which is not complementary to the corresponding base in the target.

13. The method of claim 12, wherein said base which is not complementary to the corresponding base in the target is positioned 5 or less bases from the 5' terminus.

14. The method of claim 12, wherein each of said downstream probes includes near its 5' end a base which is not complementary to the corresponding base in the target.

15. The method of claim 12, further comprising providing as the upstream probe which hybridizes with said mismatched downstream probe, a mismatched probe having near its 3' end a base which is not complementary to the corresponding base in the target, but which is complementary to the corresponding base in the complementary downstream probe.

16. The method of claim 12, further comprising performing said step b under conditions wherein the initial temperature of mixing ranges from about 50-95 °C, followed by a lower temperature to enhance annealing.

17. The method of claim 12, further comprising performing said step b under conditions wherein the concentration of monovalent cation is is greater than about 100 mM.

18. The method of claim 17, wherein said monovalent cation is selected from the group consisting of Na^+, K^+ and NR_3H^+, where R is independently selected from H or lower alkyl.

19. The method of claim 16, further comprising performing said step b under conditions wherein the concentration of monovalent cation is greater than about 100 mM.

20. A kit for detecting ΔF_{508} cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 1 and SEQ No. 2, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 3 and SEQ No. 4; or

(b) SEQ No. 5 and SEQ No. 6; or

(c) both sets (a) and (b).

21. A kit for detecting ΔF_{508} cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 1 and SEQ No. 7, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 3 and SEQ No. 4; or

(b) SEQ No. 5 and SEQ No. 6; or

(c) both sets (a) and (b).

22. A kit for detecting ΔF_{508} cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 8 and SEQ No. 9, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 3 and SEQ No. 10; or

(b) SEQ No. 5 and SEQ No. 11; or

(c) both sets (a) and (b).

23. A kit for detecting ΔF_{508} cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 14 and SEQ No. 15, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 12 and SEQ No. 13; or

(b) SEQ No. 16 and SEQ No. 17; or

(c) both sets (a) and (b).

24. A kit for detecting $G_{551}D$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the

probes are SEQ No. 24 and SEQ No. 25, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 26 and SEQ No. 27; or

(b) SEQ No. 28 and SEQ No. 29; or

(c) both sets (a) and (b).

25. A kit for detecting $G_{551}D$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 24 and SEQ No. 25, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 30 and SEQ No. 31; or

(b) SEQ No. 40 and SEQ No. 41; or

(c) both sets (a) and (b).

26. A kit for detecting $G_{551}D$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 24 and SEQ No. 25, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 32 and SEQ No. 33; or

(b) SEQ No. 42 and SEQ No. 43; or

(c) both sets (a) and (b).

27. A kit for detecting $G_{551}D$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 24 and SEQ No. 25, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 34 and SEQ No. 35; or

(b) SEQ No. 44 and SEQ No. 45; or

(c) both sets (a) and (b).

28. A kit for detecting $G_{551}D$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 24 and SEQ No. 25, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 36 and SEQ No. 37; or

(b) SEQ No. 46 and SEQ No. 47; or

(c) both sets (a) and (b).

29. A kit for detecting $G_{551}D$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 24 and SEQ No. 25, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 38 and SEQ No. 39; or

(b) SEQ No. 48 and SEQ No. 49; or

(c) both sets (a) and (b).

30. A kit for detecting $W_{1282}X$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 18 and SEQ No. 19, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 20 and SEQ No. 21; or

(b) SEQ No. 22 and SEQ No. 23; or

(c) both sets (a) and (b).

31. A kit for detecting $G_{542}X$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 62 and SEQ No. 63, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 64 and SEQ No. 65; or

(b) SEQ No. 60 and SEQ No. 61; or

(c) both sets (a) and (b).

32. A kit for detecting $G_{542}X$ cystic fibrosis mutations in a target nucleic acid the sequence of which is known, said kit comprising one or more containers holding in a suitable buffer at least four oligodeoxyribonucleotide probes, wherein two of the probes are SEQ No. 67 and SEQ No. 68, at least one of which is labeled; and the remaining probes are selected from:

(a) SEQ No. 66 and SEQ No. 69; or

(b) SEQ No. 70 and SEQ No. 71; or

(c) both sets (a) and (b).

```
16.        5′-fluorescein-GGCACCATTAAAGAAAATATCAT-3′
14.†                                                5′-TGGTGTTTCCTATGATGAATATA-biotin-3′
12.                 5′-dansyl-CACCATTAAAGAAAATATCATCTT-3′
8.†                       5′-GCACCATTAAAGAAAATATCAT-3′
5.                                                  5′-TGGTGTTTCCTATGATGAAT-3′
3.                                                5′-CTTTGGTGTTTCCTATGATGCCCGGG-3′
1.†                       5′-GCACCATTAAAGAAAATATCA-3′
    5′-TTTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAGATACAGAAGCG-3′*
72  3′-AAAAGGACCTAATACGGACCGTGGTAATTTCTTTTATAGTAGAAACCACAAAGGATACTACTTATATCTATGTCTTCGC-5′
                                                    ***
2.†                     3′-CCGTGGTAATTTCTTTTATAG-5′
4.                                              3′-AGAAACCACAAAGGATACTACCCGGG-5′
6.                                                 3′-AACCACAAAGGATACTACTT-5′
7.                      3′-CCGTGGTAATTTCTTTTATAGT-5′
9.†                      3′-CGTGGTAATTTCTTTTATAGTA-5′
10.                                             3′-GAAACCACAAAGGATACTACCCGGG-5′
11.                                                3′-ACCACAAAGGATACTACTTA-5′
13.                                             3′-GAAACCACAAAGGATACTACTTATAT-biotin-5′
15.†                    3′-CCGTGGTAATTTCTTTTATAGTA-5′
17.                                                3′-ACCACAAAGGATACTACTTATATC-biotin-5′
```

FIG.1

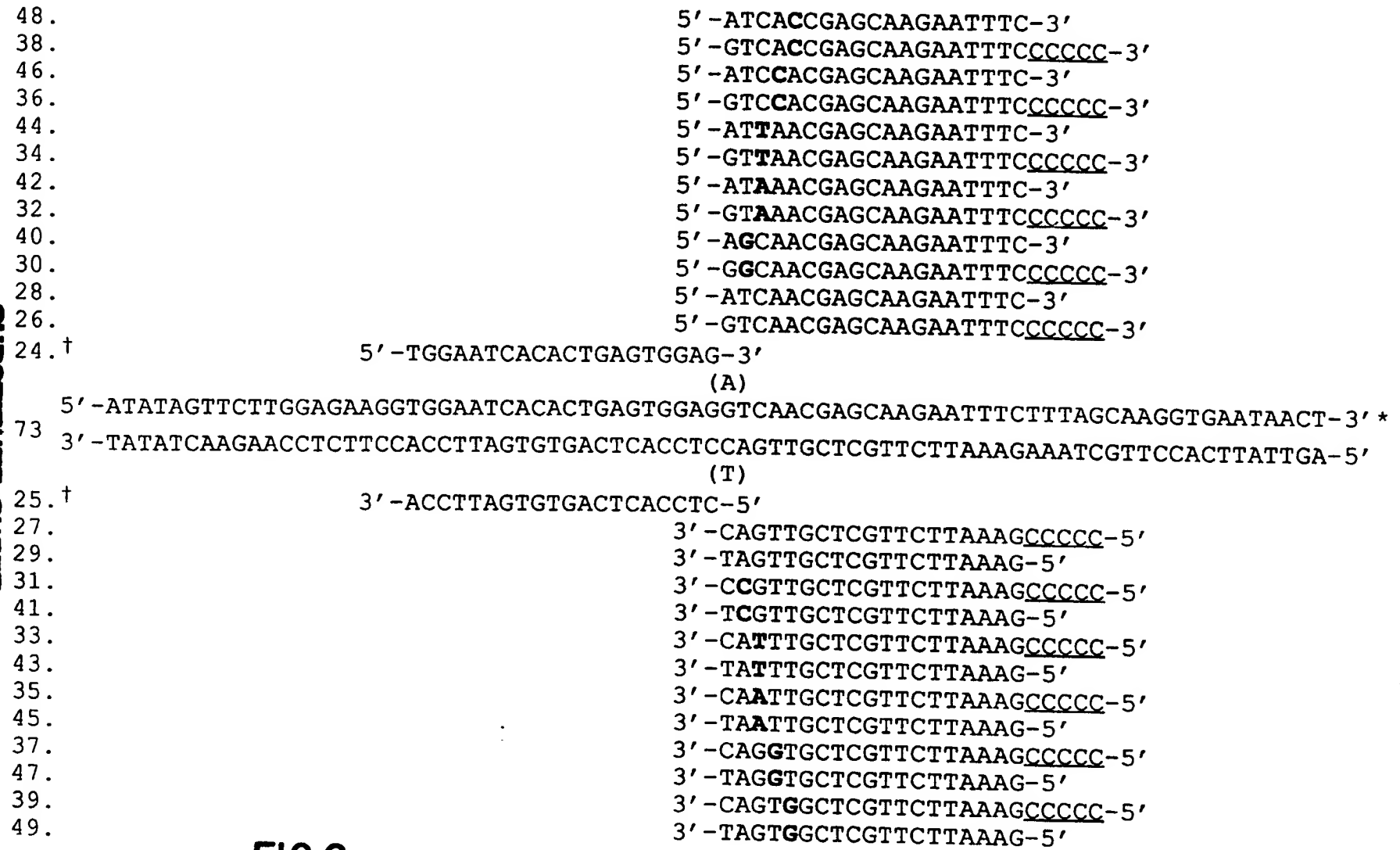
48. 5′-ATCACCGAGCAAGAATTTC-3′
38. 5′-GTCACCGAGCAAGAATTTCCCCCC-3′
46. 5′-ATCCACGAGCAAGAATTTC-3′
36. 5′-GTCCACGAGCAAGAATTTCCCCCC-3′
44. 5′-ATTAACGAGCAAGAATTTC-3′
34. 5′-GTTAACGAGCAAGAATTTCCCCCC-3′
42. 5′-ATAAACGAGCAAGAATTTC-3′
32. 5′-GTAAACGAGCAAGAATTTCCCCCC-3′
40. 5′-AGCAACGAGCAAGAATTTC-3′
30. 5′-GGCAACGAGCAAGAATTTCCCCCC-3′
28. 5′-ATCAACGAGCAAGAATTTC-3′
26. 5′-GTCAACGAGCAAGAATTTCCCCCC-3′
24.† 5′-TGGAATCACACTGAGTGGAG-3′
(A)
5′-ATATAGTTCTTGGAGAAGGTGGAATCACACTGAGTGGAGGTCAACGAGCAAGAATTTCTTTAGCAAGGTGAATAACT-3′*
73
3′-TATATCAAGAACCTCTTCCACCTTAGTGTGACTCACCTCCAGTTGCTCGTTCTTAAAGAAATCGTTCCACTTATTGA-5′
(T)
25.† 3′-ACCTTAGTGTGACTCACCTC-5′
27. 3′-CAGTTGCTCGTTCTTAAAGCCCCC-5′
29. 3′-TAGTTGCTCGTTCTTAAAG-5′
31. 3′-CCGTTGCTCGTTCTTAAAGCCCCC-5′
41. 3′-TCGTTGCTCGTTCTTAAAG-5′
33. 3′-CATTTGCTCGTTCTTAAAGCCCCC-5′
43. 3′-TATTTGCTCGTTCTTAAAG-5′
35. 3′-CAATTGCTCGTTCTTAAAGCCCCC-5′
45. 3′-TAATTGCTCGTTCTTAAAG-5′
37. 3′-CAGGTGCTCGTTCTTAAAGCCCCC-5′
47. 3′-TAGGTGCTCGTTCTTAAAG-5′
39. 3′-CAGTGGCTCGTTCTTAAAGCCCCC-5′
49. 3′-TAGTGGCTCGTTCTTAAAG-5′

FIG.2

```
                                    5'-AAGGAAAGCCTTTGGAGT-3'
                                    5'-GAGGAAAGCCTTTGGAGTCCCCC-3'
                  5'-ATTCAATAACTTTGCAACAGTG-3'
                                          (A)
      5'-ATCCAGATCGATGGTGTGTCTTGGGATTCAATAACTTTGCAACAGTGGAGGAAAGCCTTTGGAGTGATACCACAG-3'*
74    3'-TAGGTCTAGCTACCACACAGAACCCTAAGTTATTGAAACGTTGTCACCTCCTTTCGGAAACCTCACTATGGTGTC-5'
                                          (T)
                                    3'-TAAGTTATTGAAACGTTGTCAC-5'
                                          3'-CTCCTTTCGGAAACCTCACCCCCC-5'
                                          3'-TTCCTTTCGGAAACCTCA-5'
```

FIG. 3

```
70.       5'-fluorescein-AAAGACAATATAGTTCTTT-3'
68.†                                    5'-GAGAAGGTGGAATCAC-biotin-3'
66.            5'-dansyl-AAAGACAATATAGTTCTTG-3'
64.            5'-dansyl-AAAGACAATATAGTTCGTG-3'
62.†                                    5'-GAGAAGGTGGAATCA-biotin-3'
60.       5'-fluorescein-AAAGACAATATAGTTCCTT-3'
59.            5'-TGCAGAGAAAGACAATATAGTTCTTTGAGAAGGTGGAATCACACTGAGTGG-3'
58.            5'-TGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATCACACTGAGTGG-3'
                                          (T)
     5'-TCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATCACACTGAGTGGAGGTCAACGAGCAAGAATT-3'*
75   3'-AGAGGTTCAAACGTCTCTTTCTGTTATATCAAGAACCTCTTCCACCTTAGTGTGACTCACCTCCAGTTGCTCGTTCTTAA-5'
                                          (A)
61.                  3'-TTTCTGTTATATCAAGGAA-5'
63.†                                    3'-CTCTTCCACCTTAGT-biotin-5'
65.                  3'-TTTCTGTTATATCAAGCAC-5'
67.†                3'-CTTTCTGTTATATCAAGAA-5'
69.                                   3'-CCTCTTCCACCTTAGT-biotin-5'
71.                                   3'-ACTCTTCCACCTTAGT-biotin-5'
```

FIG. 4

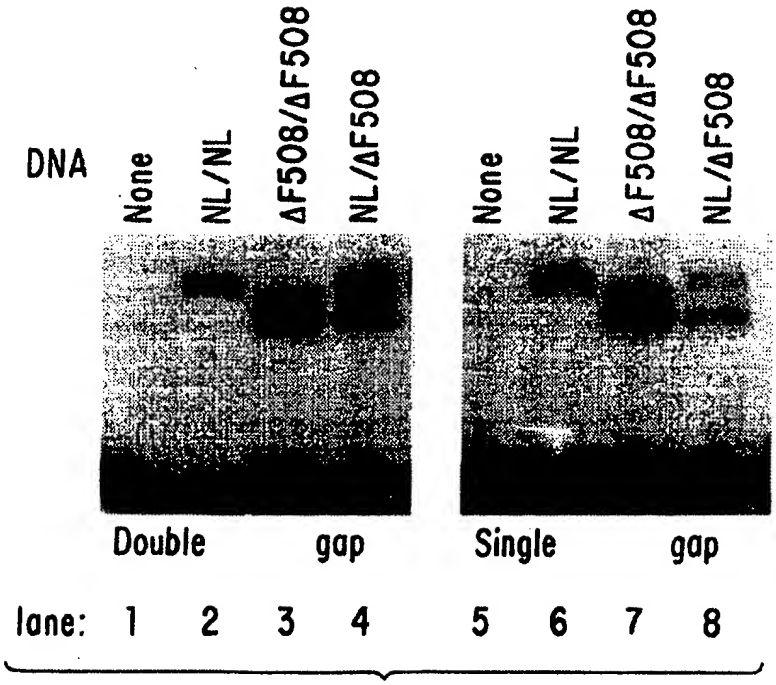
DNA
None
NL/NL
ΔF508/ΔF508
NL/ΔF508
None
NL/NL
ΔF508/ΔF508
NL/ΔF508
Double gap
Single gap
lane: 1 2 3 4 5 6 7 8

FIG. 5

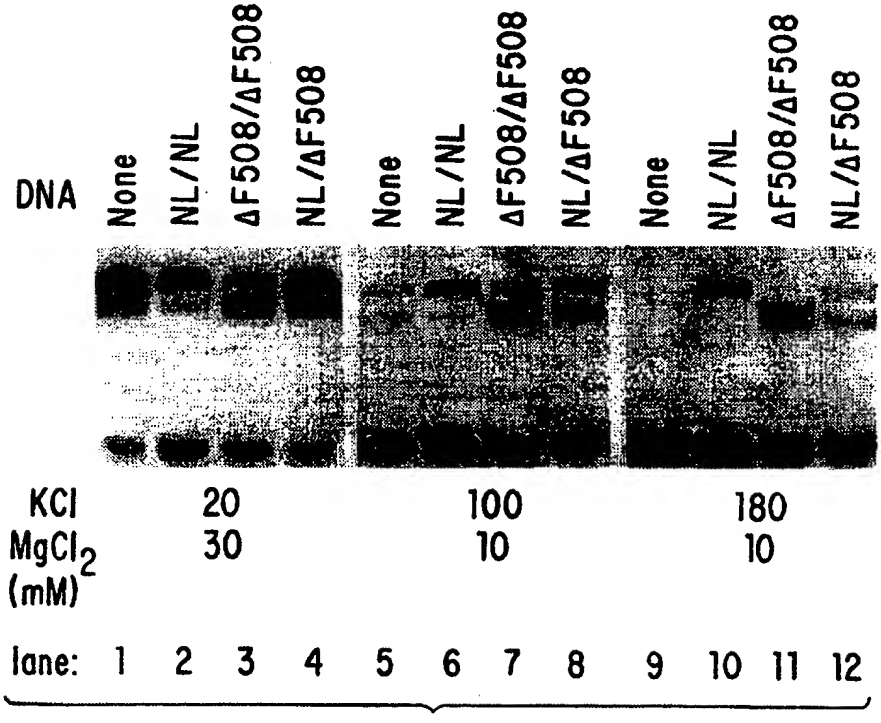
DNA
None
NL/NL
ΔF508/ΔF508
NL/ΔF508
None
NL/NL
ΔF508/ΔF508
NL/ΔF508
None
NL/NL
ΔF508/ΔF508
NL/ΔF508
KCl 20 100 180
MgCl2 30 10 10
(mM)
lane: 1 2 3 4 5 6 7 8 9 10 11 12

FIG. 6

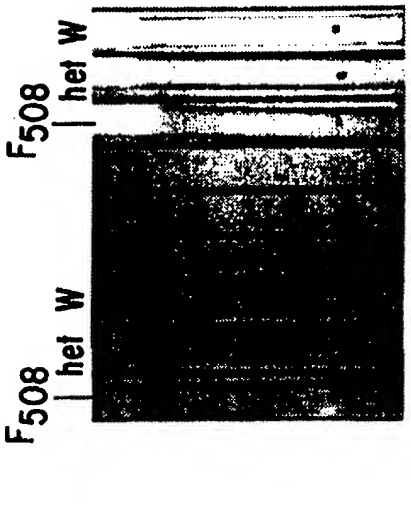

FIG. 7c

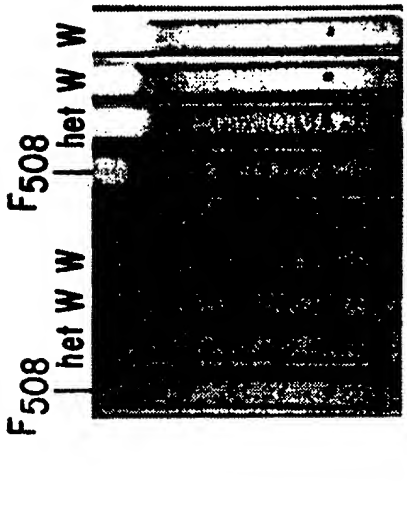

FIG. 7b

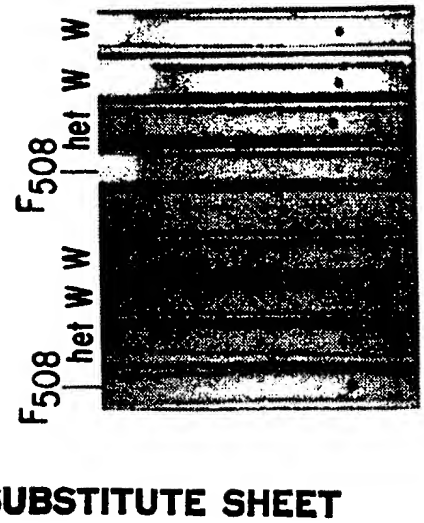

FIG. 7a

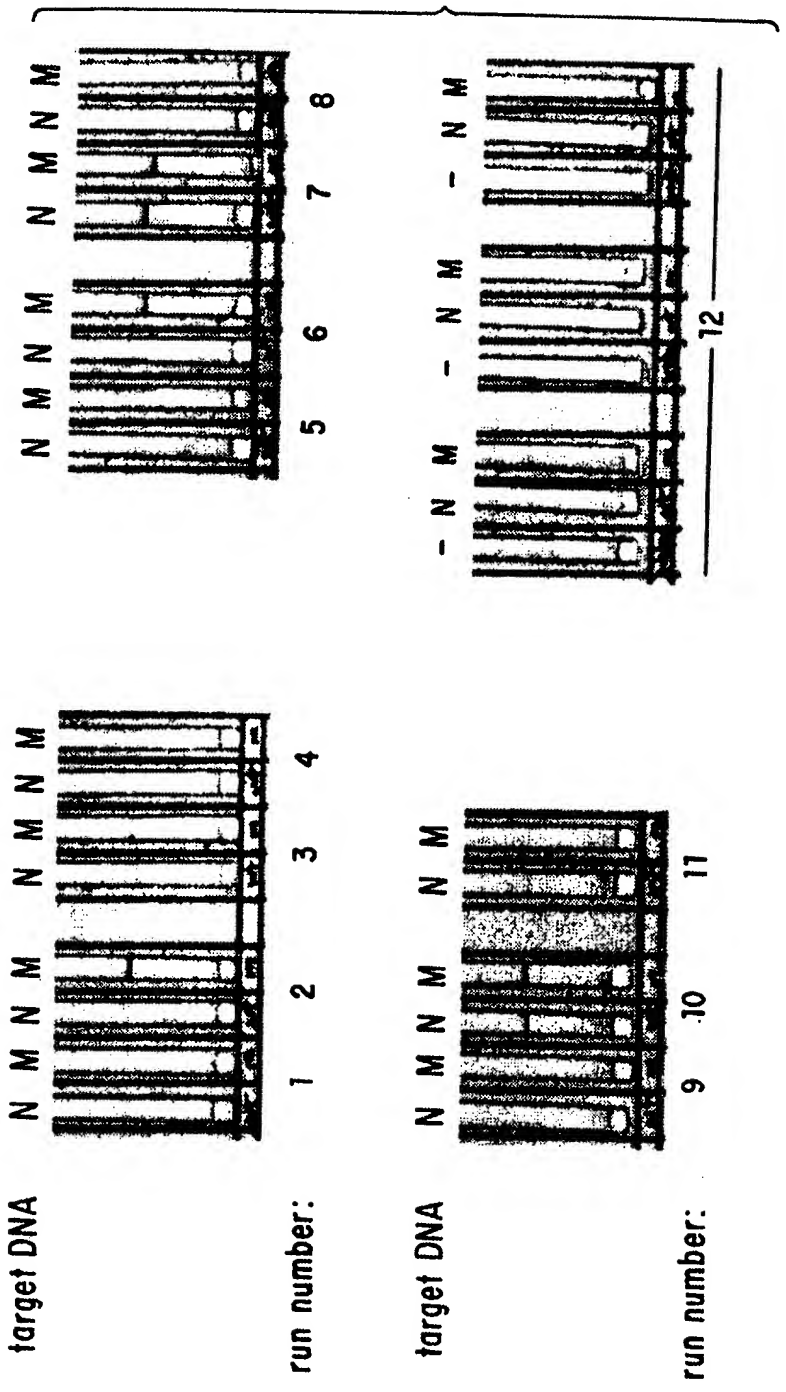
target DNA
run number:
N M N M N M N M
1 2 3 4
target DNA
run number:
N M N M N M
9 10 11
N M N M N M N M
5 6 7 8
- N M - N M - N M
12

FIG. 8

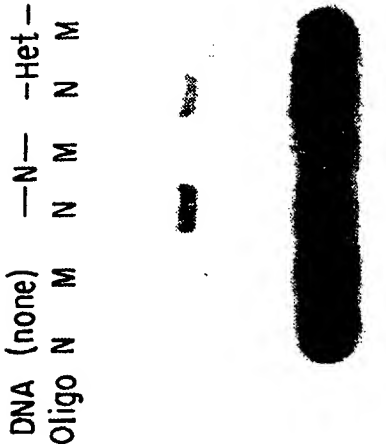

FIG. 9b

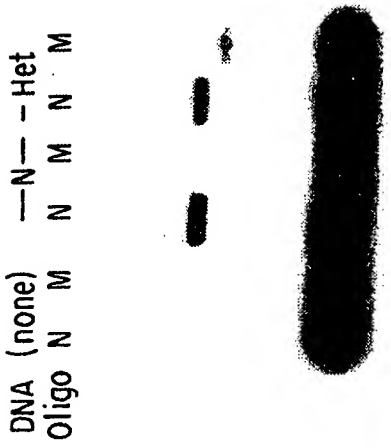

FIG. 9a

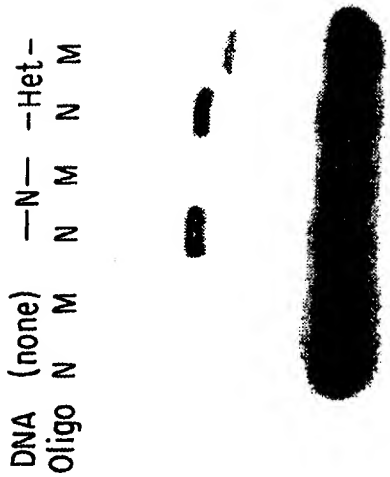

FIG. 9d

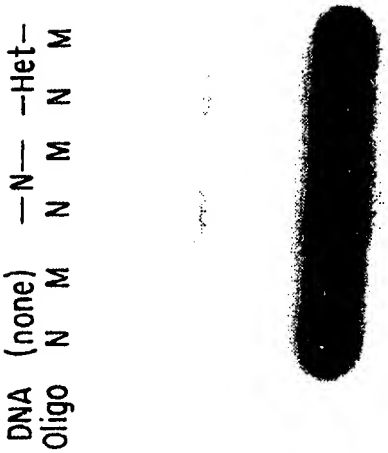

FIG. 9c

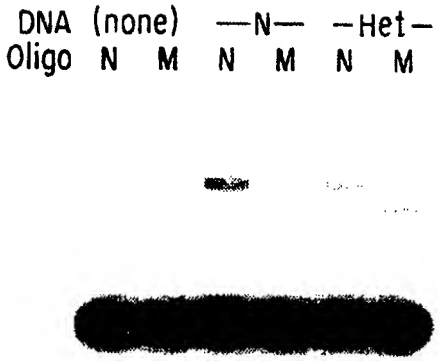

FIG. 9e

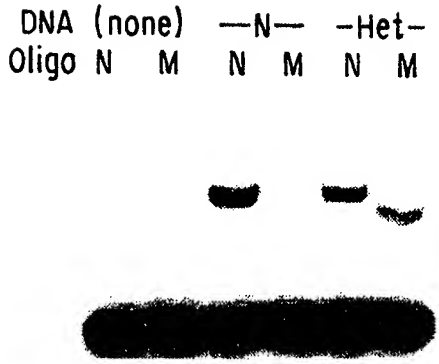

FIG. 9f

```
56.  5'-dansyl-ACCTTATTGCTCCCCTTTACC-3'
54.                          5'-GAATGCTGGTCCTGACAC-biotin-3'
52.  5'-dansyl-ACCTTATTGCTCCCCTTTCCC-3'
                                (T)
          (5'-TGACCTTATTGCTCCCCTTTCCCGGAATGCTGGTCCTGACACAACAATAT-3')
50.         3'-ACTGGAATAACGAGGGGAAAGGGCCTTACGACCAGGACTGTGTTGTTATA-5'
51.         3'-ACTGGAATAACGAGGGGAAAGGACCTTACGACCAGGACTGTGTTGTTATA-5'
53.           3'-TGGAATAACGAGGGGAAAGG-5'
55.                             3'-CCTTACGACCAGGACTGTG-biotin-5'
57.           3'-TGGAATAACGAGGGGAAATG-5'
```

FIG. 10

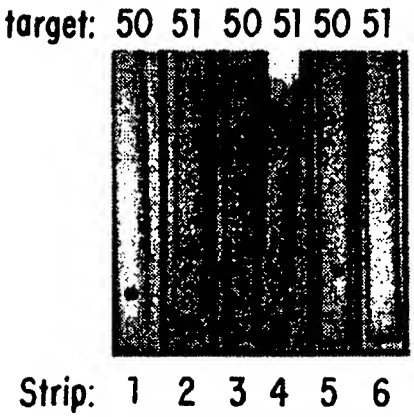

FIG. 11

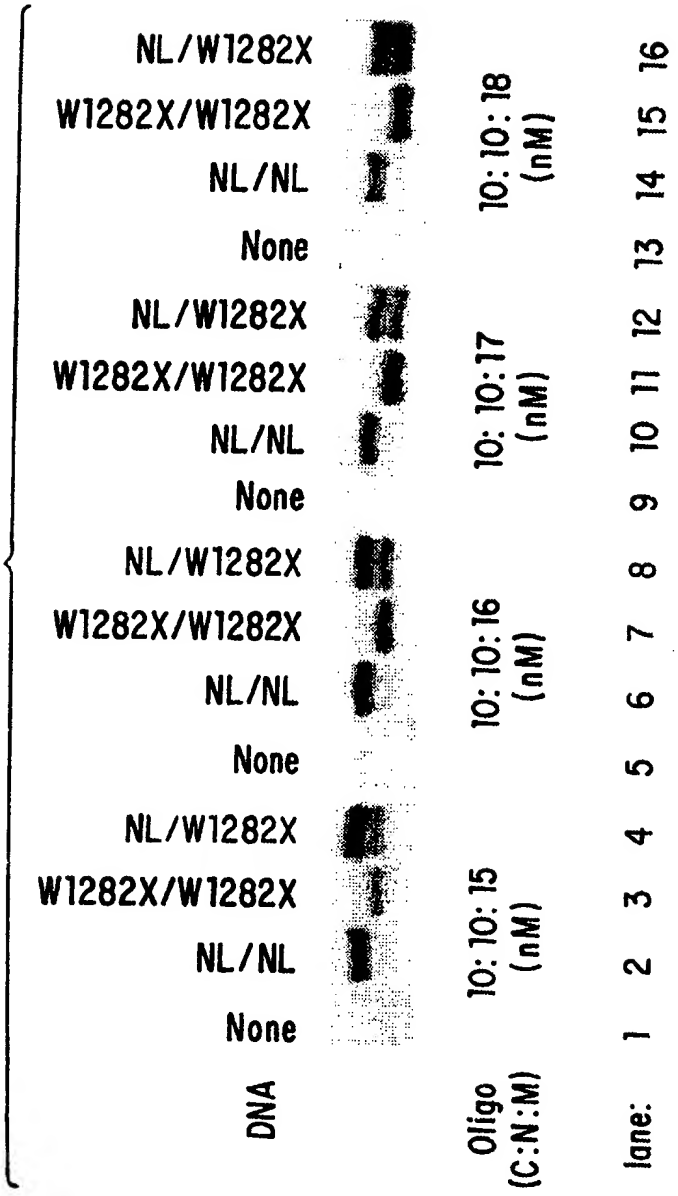
DNA
NL/W1282X
W1282X/W1282X
NL/NL
None
NL/W1282X
W1282X/W1282X
NL/NL
None
NL/W1282X
W1282X/W1282X
NL/NL
None
NL/W1282X
W1282X/W1282X
NL/NL
None
Oligo (C:N:M)
10:10:15 (nM)
10:10:16 (nM)
10:10:17 (nM)
10:10:18 (nM)
lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 12

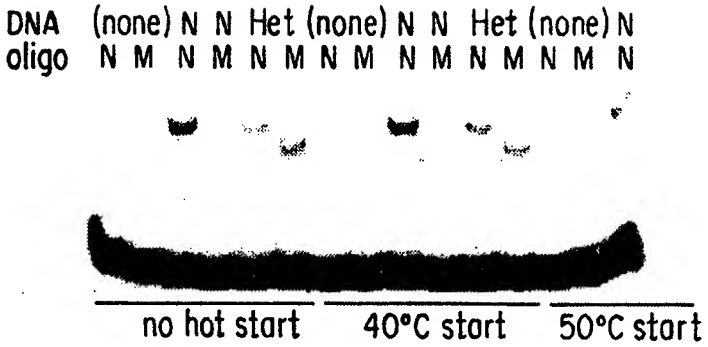

FIG. 13a

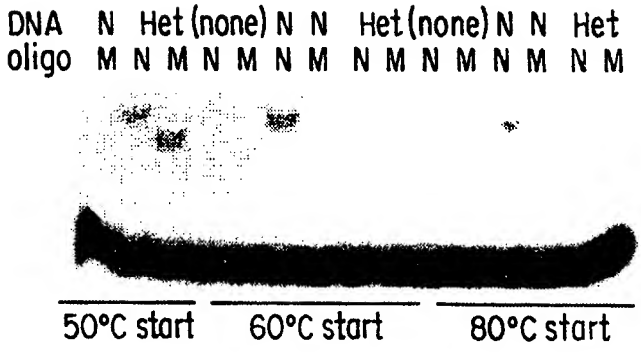

FIG. 13b

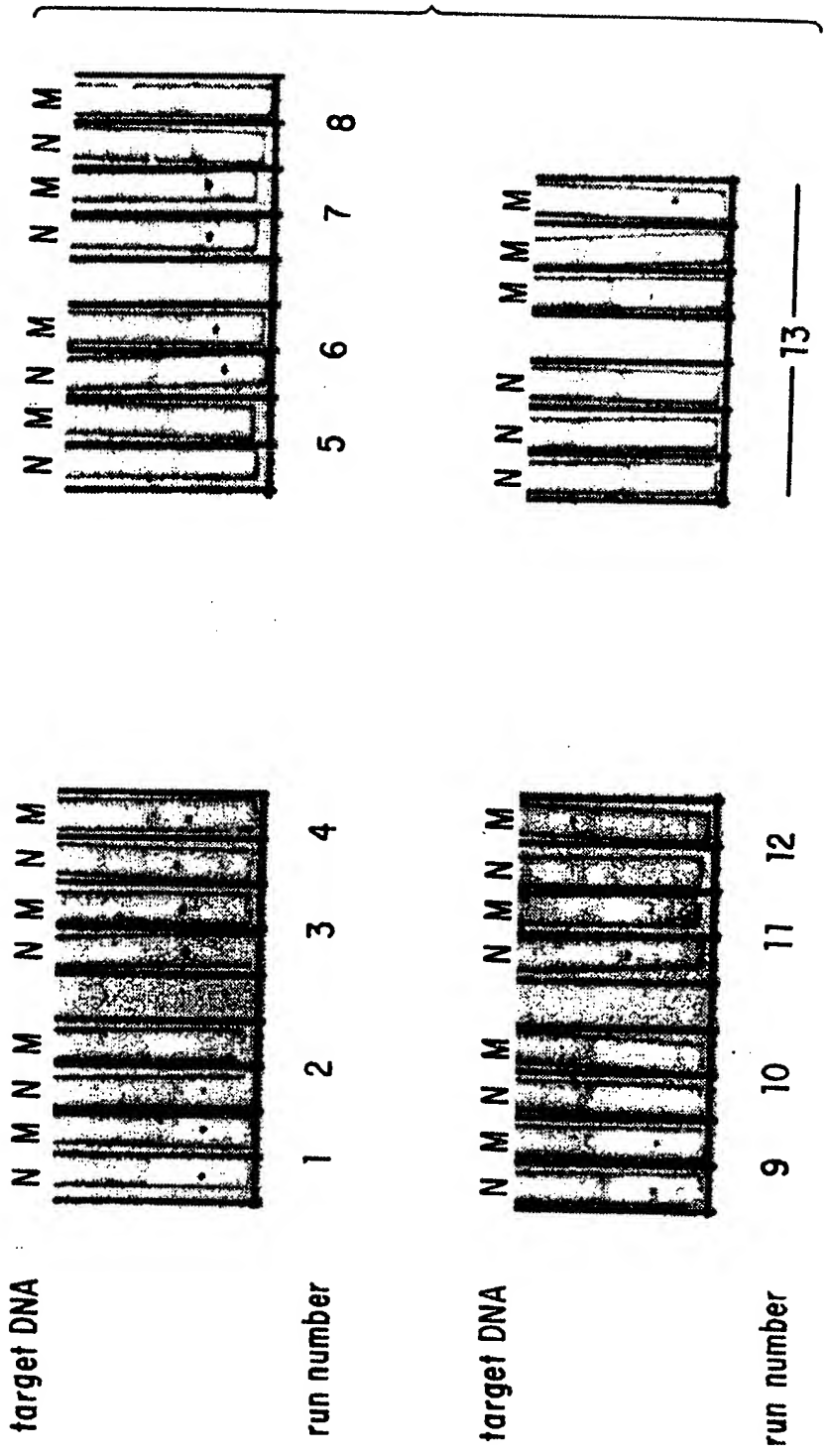

FIG. 14a

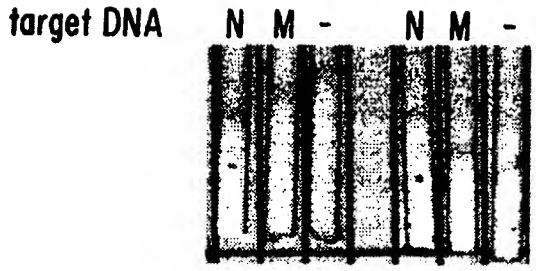

FIG. 14b